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SECTION MEETINGS

ILLINOIS	
University of Chicago	March 9, 1943
NEW YORK	
New York Academy of Medicine	March 17, 1943
WISCONSIN	
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14113 P

Use of Highly Purified Rations in the Study of Nicotinic Acid Deficiency.

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The corn meal used in modified Goldberger diets[1,2] for the production of canine blacktongue provides the animals with 0.1-0.12 mg per kilo per day of nicotinic acid.[3,4] Dogs ingesting such diets require, in addition, approximately 0.1 mg of nicotinic acid per kilo per day.[5] This agrees with the finding[6] that adult dogs require 0.2-0.225 mg per kilo per day of nicotinic acid when fed a purified diet low in nicotinic acid. Thus half the requirement of each dog is provided by the corn meal of the Goldberger diets.

Almost all dogs fed a modified Goldberger diet[1] have become acutely ill within 75 days and most of them have fallen ill within 40 days. It was thought that if dogs were fed a purified ration in which the nicotinic acid supplied was reduced to a minimum, the appearance of blacktongue should be considerably accelerated. The object was to continue the study of the physiological defects in acute deficiency rather than in intermediate stages.

Eleven adult mongrel dogs were fed a purified ration whose nicotinic acid content was held almost to its minimum possible level, less than 0.01 mg per kilo per day. The diet was identical with that of Schaeffer, McKibbin and Elvehjem[7] except that corn starch was substituted for half of the sucrose component. Each dog was housed in an individual cage; food, water intake and urine were measured daily.

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[1] Kohn, H. I., Klein, J. R., and Dann, W. J., *Biochem. J.*, 1939, **33**, 1432.

[2] Koehn, D. J., and Elvehjem, C. A., *J. Biol. Chem.*, 1937, **118**, 693.

[3] Teply, L. J., Strong, F. M., and Elvehjem, C. A., *J. Nutrition*, 1942, **23**, 417.

[4] Sarett, H. P., *J. Nutrition*, 1942, **23**, 35.

[5] Margolis, G., Margolis, L. H., and Smith, S. G., *J. Nutrition*, 1938, **16**, 541.

[6] Schaeffer, A. E., McKibbin, J. M., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **144**, 679.

[7] Schaeffer, A. D., McKibbin, J. M., and Elvehjem, C. A., *J. Nutrition*, 1942, **23**, 491.

Since no dog fell ill until the 63rd day, there were, seemingly, no advantages in using this costly diet. Four dogs became acutely ill after 63, 78, 85 and 98 days respectively, as judged by weight loss, anorexia, bloody diarrhea, thick, ropy salivation and the appearance of necrotic lesions of the cheeks, gums and throat. However, their state did not completely resemble that of blacktongue dogs on the Goldberger diet. Despite the acuteness of the deficiency syndrome the animals were not prostrate; they were alert and able to exercise. Moreover, each dog drank at least 500 cc of water daily, while dogs in blacktongue produced by Goldberger diets do not drink appreciable quantities of water despite their profound dehydration. There was no anuria, hemoconcentration or other evidence of dehydration although in all other respects the animals agreed with our concept of the nicotinic acid deficiency syndrome.

After 8 to 14 days in this condition, in which no therapeutic steps were taken, each dog spontaneously improved and 10 days later there was no evidence of blacktongue. Their subsequent behavior was identical with the description of blacktongue dogs produced on the Goldberger diet and treated with salt.[8] Appetite, weight and hemoglobin declined steadily.

Four dogs developed acute blacktongue on this ration after 89, 94, 145 and 187 days, respectively, and died within one week. Their condition was identical in all respects with that of the previous 4 dogs. They showed the classical signs of blacktongue but were not anuric, drank copious quantities of water and were not dehydrated or prostrate until a few hours before death. Their blood hemoglobin concentrations declined from original values of 15.2, 15.1, 14.8 and 13.5 g % to 12.3, 10.5, 10.0 and 7.4 g % respectively just before acute blacktongue and these levels did not rise appreciably during blacktongue. Their liver nicotinic acid concentrations were within the range found for blacktongue dogs on a Goldberger diet.[9] At autopsy the usual lesions were noted[10] and 2 showed signs of pneumonia which was probably due to aspiration of the Vincent's organisms which are responsible for the mouth lesions.[11]

The remaining 3 animals never developed blacktongue. For 5 months their appetites, body weight and blood hemoglobin steadily diminished. Thus, they resembled nicotinic acid deficient dogs who have been successfully treated with physiological saline.[8] One dog was permitted to decline and finally die in this deficient state. Just before death the animal had lost 65% of its weight and showed a hemoglobin concentration of 1.6 g %, hematocrit of 7% and red cell count of 1,020,000 per mm.[3] The other 2 dogs were not permitted to decline this far and were saved for a study of the nature of the anemia.

This inconsistent behavior of dogs on a purified ration makes it difficult to evaluate the nature of the physiological defects in nicotinic acid deficiency. Certainly, their behavior is in marked contrast to that observed earlier on Goldberger diets.[8] Since the dehydration, lack of thirst and hemoconcentration characteristic of blacktongue produced by the ingestion of corn-meal-containing Goldberger diets are also seen in human pellagra, it seems not unlikely that the presence of corn meal *per se* may be an etiological factor in pellagra.

The author's thanks are due to the John and Mary R. Markle Foundation for its support of this work and to Merck and Co., Rahway, N.J., for a supply of the crystalline vitamins used in this study.

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[8] Handler, P., and Dann, W. J., *J. Biol. Chem.*, 1942, **145**, 145.

[9] Dann, W. J., and Handler, P., *J. Nutrition*, 1941, **22**, 409.

[10] Lillie, R. D., *Nat. Inst. of Health Bull.*, 1933, **162**, 13.

[11] Smith, D. T., Persons, E. L., and Harvey, H. I., *J. Nutrition*, 1937, **14**, 373.

14114 P

Minimum Vitamin A Requirements in Infants as Determined by Vitamin A Concentration in Blood.

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In previous communications[1] we reported that the vitamin A concentration in the blood is a good indicator of the size of the vitamin A stores in the rat. Thus, low levels of the vitamin in the blood were usually associated with little or no storage in the liver, the chief depot of vitamin A, whereas high concentrations in the blood were accompanied by large stores. Intermediate blood values were of less significance as they were associated with either small or large amounts of vitamin A in the liver.

Studies carried out in infants[2] coincided, in general, with the results of our experiments in rats and indicated the practical importance of the vitamin A blood level in the diagnosis of vitamin A deficiency. In a series of 93 normal infants, ranging in age from 3 weeks to 6 months, the lowest concentration of vitamin A in the blood was found to be 45 international units per 100 cc plasma. Further observations have indicated that 40 international units may be regarded as the lower limit of the normal range of the vitamin A concentration of the blood for infants under 6 months of age. Values below this level were obtained in infants who, either were suffering from disorders interfering with the absorption of the vitamin as, for example, cystic fibrosis of the pancreas, coeliac disease and congenital obliteration of the bile ducts, or were receiving diets devoid of vitamin A.

The present study was undertaken to determine the daily minimum intake of vitamin A required to bring about normal blood levels in young infants having low concentrations of the vitamin in the blood as a result of a diet devoid of vitamin A. It was thought that an investigation of this kind might be of value in ascertaining the minimum vitamin A requirements in infants. In a previous communication[3] we found that, as judged by dark adaptation, the minimum vitamin A requirement for infants was 25 international units per kilogram of body weight. As a more recent study[4] revealed that the vitamin A blood level is a more sensitive indicator of vitamin A deficiency than is dark adaptation, the possibility arose that the requirements might be higher if the former were used as a criterion of vitamin A adequacy.

Daily supplements of 150, 600 and 1200 units of vitamin A in the form of U.S.P. reference cod liver oil were given to 12 infants who had subnormal blood levels following the administration of a diet devoid of vitamin A. Also included in this study is an infant (A.I.) who had been receiving a vitamin A-free diet for several weeks and who had a blood vitamin A level within the normal zone, *i.e.* 48 units. Six hundred units were given daily to this baby to determine whether the blood level could be maintained in the normal range at this level of vitamin A intake. After the supplements were given for a period of 4 weeks, the concentration of vitamin A in the blood was determined by a method which was fully described elsewhere.[2]

The effect of these supplements on the vitamin A blood levels and, in several instances, on dark adaptation, may be observed in the accompanying table. It will be noted that a daily intake of 150 and 600

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* Captain, Army Medical Corps, Medical Department Research Laboratory, Edgewood Arsenal, Maryland.

[1] (a) Lewis, J. M., Bodansky, O., Falk, K. G., and McGuire, G., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 248; (b) Lewis, J. M., Bodansky, O., Falk, K. G., and McGuire, G., *J. Nutrition*, 1942, **23**, 351.

[2] Lewis, J. M., Bodansky, O., and Haig, C., *Am. J. Dis. Child.*, 1941, **62**, 1129.

[3] Lewis, J. M., and Haig, C., *J. Pediatrics*, 1939, **15**, 812.

[4] Bodansky, O., Lewis, J. M., and Haig, C., *Science*, 1941, **94**, 370.

TABLE I.
Effect of Varying Supplements of Vitamin A on Concentration of the Vitamin in Blood and on Dark Adaptation in Infants Previously Receiving a Diet Devoid of Vitamin A.

				Vit. A in blood plasma units per 100 cc		Final rod threshold log micromicro-Lamberts*	
Subject	Age, mo.	Weight, kg	Units of Vit. A daily for 4 weeks	Prior to supplement	After supplement	Prior to supplement	After supplement
J.K.	3½	6.2	150	13	16	4.4	3.3
N.R.	5½	6	150	11	16	5.0	3.0
J.R.	6	8.3	150	23	35	4.7	3.3
L.R.	3¾	5.8	600	30	26	3.8	3.0
J.K.	4½	6.7	600	16	17		
N.R.	6½	6.4	600	16	32		
J.R.	7	9	600	35	28		
A.I.	4⅓	6	600	48	32		
S.K.	3¼	5	1200	30	41		
J.R.	3½	5	1200	16	41		
L.G.	4	5.7	1200	27	41		
G.A.	3	5.5	1200	37	53		
M.S.	5	6.2	1200	33	54		

* Final rod thresholds higher than 3.3 are abnormal.[3]

units for 4 weeks had no appreciable effect on the vitamin A concentration in the blood whereas 1200 units brought the blood concentration into the normal range in the 5 infants receiving this dosage. In the infant whose blood concentration was normal (48 units) prior to the administration of 600 units the blood level dropped to 32 units at the end of 4 weeks. Thus, an intake of 600 units not only failed to raise the blood levels to the normal range in 4 infants but also was unable to maintain the normal blood level in one infant. Although the administration of 150 units of vitamin A did not increase the blood concentration, this unitage, as previously reported,[4] was adequate to restore dark adaptation to normal.

The results of this study indicate that, when the blood concentration is employed as the criterion of vitamin A adequacy, the daily minimum requirement for infants under 7 months of age lies somewhere between 600 units, or 100 units per kg body weight, and 1200 units, or 200 units per kg body weight. It is probable, however, that the requirement is much closer to the latter value since this amount raised the plasma concentration to values, 41-54 units per 100 cc, which were just within the normal range, 40-114 units per 100 cc.[2] Thus, more than 4 times and probably close to 8 times as many units are required when the blood level of vitamin A rather than dark adaptation is used as the basis for estimating the vitamin A status of the infant.

14115 P

Cytoplasmic Changes in Rous Sarcoma Cells Cultivated *in vitro*.

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We have previously reported some nuclear alterations in Rous sarcoma cells grown in pure culture.[1] The following is a brief account of the cytoplasmic changes which are generally developed in Rous sarcoma cells cultivated *in vitro*.

The most outstanding changes take place in the central area of the cell. The cytoplasm of the central cell region at first takes on a cloudy appearance. Indefinite at first, this zone gradually becomes better defined and finally assumes a very sharp outline (Fig. 1). In general, the segregated central area is 3-6 times as large as the nucleus, is spherical in shape and always appears to be denser than the surrounding cytoplasm, but shows no pronounced structural peculiarities. In the first stage of its development the central area is always somewhat eosinophilic; in the more advanced stage of the segregation process its tinctorial characteristics become rather indefinite; with Giemsa it stains at times slightly red and at times slightly blue, and mostly in mixed tones. The nucleus lies almost invariably outside the central area; in

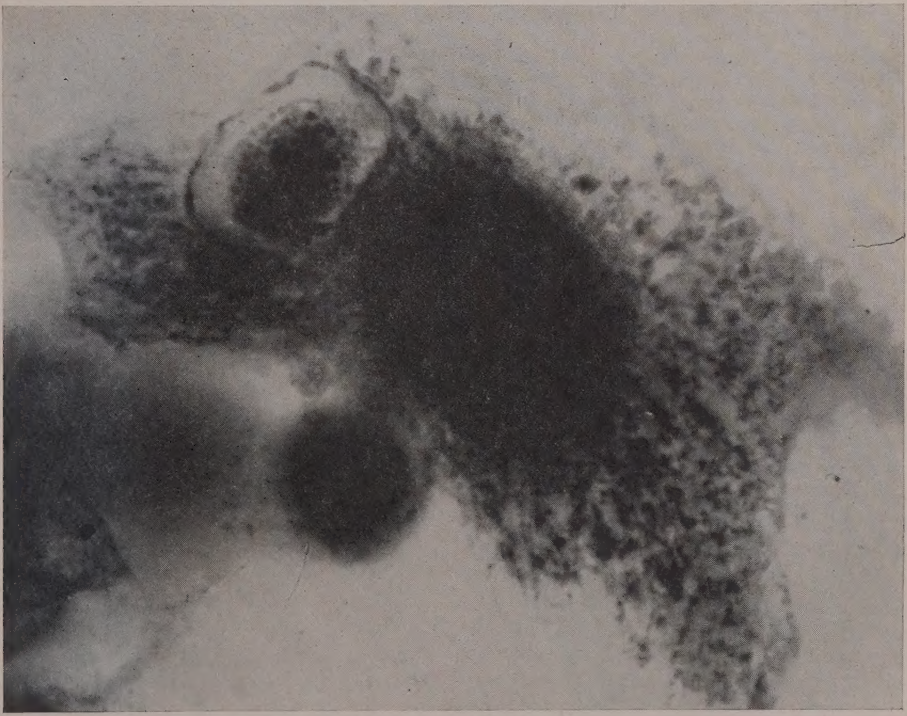

Fig. 1.
Rous sarcoma cells; culture No. 12711; Giemsa's stain. × 1790.

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[1] Tenenbaum, E., and Doljanski, L., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 236.

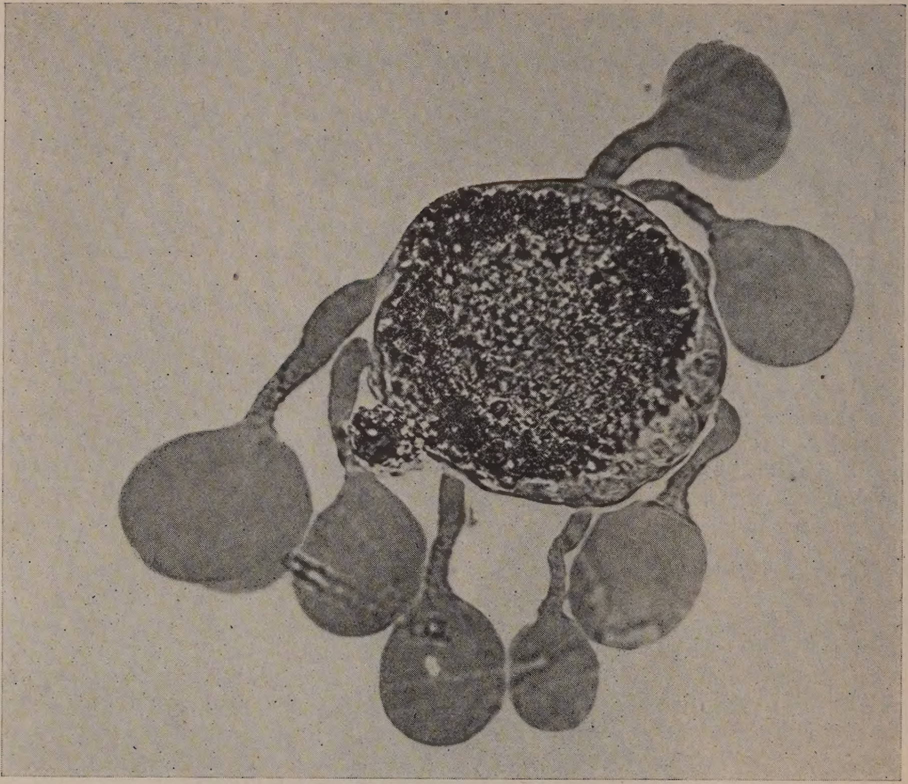

FIG. 2.
Rous sarcoma cell; culture No. 11764; living. × 585.

binuclear cells the central area is situated between the two nuclei.

As the result of the segregation of the central cell region the sarcoma cell often appears to be built of two clearly distinct zones—central area and peripheral cytoplasm. The peripheral cytoplasm of the sarcoma cell involved is not only sharply marked off from the central region, but in itself presents striking structural and behavioral peculiarities. It possesses the character of a flat membrane constantly changing in outline. The vigorous movement of this membrane never leads to significant displacement of the cell. Pseudopodia projected by these cells have a very unusual aspect (Fig. 2). They often appear as coarse rigid rods, which move in all directions on their base, as on a joint. The distal ends of these pseudopodia sometimes swell and become spherical. The protoplasmic balls so formed float on their flexible stems, and often become detached.

In correspondence with the heterogeneity of structure of the cytoplasm of the sarcoma cells, the distribution of the vacuoles is not diffuse but regional, being restricted often to the boundary region between both. The vacuoles are usually numerous; some contain fat while others are devoid of fat. With neutral red, the vacuolar system of the sarcoma cells can be clearly demonstrated.

Often the peripheral cytoplasm of the sarcoma cell contains a close network of thick or thin threads and granules which stain intense blue with Giemsa and display the aspect of hypertrophic mitochondria. Frequently, small eosinophilic granules are found scattered throughout the sarcoma cells. Here and there this eosinophilic material is also found in the form of large homogeneous clumps, which are occasionally enclosed in a vacuole. Accumulations of eosinophilic material bearing a great resemblance to the intracellular eosinophilic masses may at times also be found extracellularly.

The cytoplasmic changes described above occur simultaneously with the nuclear alterations previously noted by us in sarcoma cells maintained in pure culture. Both are accompanied by great and sometimes extreme hypertrophy of the cells involved.

Our observations indicate that the peculiar nuclear and cytoplasmic alterations which we have described, and which have also been pointed out in part by earlier investigators (Borrel, Fischer, Roskin, Zweibaum), represent different aspects of a slowly progressive and decidedly pathological process. Although the changes in the sarcoma cells are extremely varied and complex their combination forms a definite syndrome of serious cell disease which, in conditions of life *in vitro*, inevitably overtakes cells bearing the causative agent of Rous sarcoma, and leads ultimately to the death of the sarcoma cell colonies.

14116

Presence in Various Grains of Factors Inhibiting Tumor Growth.

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The following report deals with the results of experiments on the influence of various grains on tumor growth. These studies were stimulated by experiments of Lewisohn and coworkers.[1] They observed that the therapeutic results of intravenous therapy of yeast extracts on spontaneous mammary carcinomas in mice could be improved by feeding polished rice instead of a normal diet. The question was raised, whether this improvement was due to a lack of substances in the polished rice diet essential for tumor growth, or whether the polished rice contains an additional tumor inhibitor. To study this problem in detail, we employed a rapid test for tumor growth inhibitors, developed in this laboratory by Laszlo and Leuchtenberger.[2]

Method. In the rapid test, treated groups of transplanted tumor mice (Sa.180) are compared with untreated controls as to the size and weight of their tumors. At the start of the experiment, 7-10 days after transplantation, groups of 7 mice are formed, each bearing a transplanted Sa.180 of the same size from the same transplant. If these matched groups are injected intravenously twice daily with saline solution for 2 consecutive days, the tumor-sizes and tumor-weights of treated animals and controls will agree after 48 hours. If one or more of these groups are injected intravenously twice daily on 2 consecutive days with the standard preparation (yeast extract as previously described) the tumor growth in these groups is inhibited. The extent of this inhibition is such that after 48 hours the differences of mean terminal size and mean terminal weight in treated and untreated groups permit the detection of tumor growth inhibitors even in a small number of animals.

Results. In a series of experiments it was found that at least four intravenous injections of 0.1 cc (corresponding to 5.25 mg solids) of the standard are necessary for an inhibitory effect, which is statistically significant, if mice are fed a normal diet. If mice are fed a polished rice diet, 4 injections of 1.75 mg solids of the same standard are sufficient for a similar inhibition of tumor growth. Control groups

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[1] Lewisohn, R., Leuchtenberger, C., Leuchtenberger, R., Laszlo, D., and Dische, Z., *Cancer Research*, 1942, **2**, 818.

[2] Laszlo, D., and Leuchtenberger, C., to be published in *Cancer Research*, 1943.

TABLE I.

Effect of a Standard Preparation on Tumor Growth in Mice on Normal Diet and on Polished Rice Diet.

Female Rockland mice transplanted with Sarcoma 180 on June 6, 1942, passage MP24. Four intravenous injections (2 on June 15 and 2 on June 16).

			June 15		June 17		June 17	
No. of animals	Diet	Dose of standard in mg	Mean initial size	Standard error	Mean terminal size	Standard error	Mean terminal size	Standard error
7	Mouse pellets	5.25	.159 in^2	.011 in^2	.121 in^2	.017 in^2	220 mg	45 mg
7	,, ,,	1.75	.169	.012	.214	.024	489	69
7	,, ,,	0 (Inj. saline sol.)	.173	.013	.229	.029	524	111
7	Polished rice	1.75	.169	.014	.149	.022	169	30
7	,, ,,	0 (Inj. saline sol.)	.163	.015	.229	.023	499	68

with saline injections kept on polished rice diet do not differ materially in their tumor growth from control groups kept on normal diet. Table I illustrates one such experiment.

Similar results were obtained in a set of experiments which are presented in Table II (1-5).

To study whether these results were caused by the deficiency in the polished rice diet, the following experiments were carried out (Table II):

(a) Mice were fed synthetic diets with* or without† the addition of vitamins of the B complex and 4 injections of 1.7 mg of the standard were given. The results did not differ from those obtained in animals injected with the same dose and kept on normal diet (Table II, 23-26).

(b) Mice were fed polished rice with 3% casein. The results were similar to those obtained with polished rice alone (Table II, 10).

(c) Mice were fed polished rice and the vitamins of the B complex. The feeding of these vitamins did not change the results materially (Table II, 11).

(d) Mice were fed brown rice instead of polished rice and injected intravenously with 1.7 mg of the standard. Inhibition of tumor growth in the mice fed brown rice was similar to the growth inhibition in animals fed polished rice (Table II, 7).

(e) Mice were fed rice bran diet and injected intravenously with 1.75 mg of the standard. The tumors appeared to grow like those in mice on normal diet. Autoclaving of the rice bran for 4 hours at 120°C seems to stimulate tumor growth (Table II, 8 and 9).

(f) Mice were fed polished rice previously autoclaved for four hours at 120°C and injected intravenously with 1.7 mg of the standard. The inhibitory effect of the polished rice was lost (Table II, 6).

From these observations it seems evident that the increased inhibitory effect, resulting from the combined polished rice diet and intravenous injection of the standard, is not due to dietary deficiency. Nor is the effect apparently caused by caloric restriction as determination of food consumption and body weight in experiments with other deficient diets indicate. Neither loss of body weight nor decreased food intake explain this effect. However, these experiments suggest the presence of a tumor growth-inhibiting factor in the rice. Therefore, the extraction of a tumor growth inhibitor from the rice was attempted. A watery extract was prepared which, when injected intravenously in a dose of 0.1 cc corresponding to 2.4 mg solids, inhibits tumor growth to a similar extent as 5.25 mg of the standard, both groups being fed on normal

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* Synthetic diet with the addition of vitamins: I. Sucrose, 75%; casein, 18%; salts, 5%; cod liver oil, 1%; corn oil, 1%; thiamin, 200 γ; riboflavin, 500 γ; nicotinamid, 1 mg; alanin, 2 mg; cholin, 1.7 mg; pantothenic acid, 1 mg; pyridoxine, 200 γ.

† Synthetic diets without the addition of vitamins: II. Casein, 3.9%; starch, 87%; cod liver oil, 3%; salts (modified Osborne-Mendel), 6%. III. Casein, 3.9%; starch, 65%; crisco, 22%; cod liver oil, 3%; salts (modified Osborne-Mendel), 6%.

TABLE II.
Effect of a Standard Preparation on Tumor Growth in Mice Fed Different Diets.

Serial No.	No. of animals	No. of exp.	Dose of standard, mg	Diet	Mean terminal tumor wt, mg	Standard error
				Normal		
1	41	6	5.25	Mouse pellets	254.8	17.7
2	28	4	1.75	” ”	523.4	43.8
3	89	13	0	” ”	548.8	23.0
				Rice		
4	141	21	1.75	Polished rice	220.8	9.5
5	122	18	0	” ”	558.8	19.4
6	20	3	1.75	Autocl. polished rice	477.0	38.3
7	20	3	1.75	Brown rice	258.0	30.4
8	21	3	1.75	Rice bran	458.7	47.9
9	7	1	1.75	Autocl. rice bran	682.9	123.4
10	7	1	1.75	Polished rice + 3% casein	221.4	63.9
11	7	1	1.75	Polished rice + Vit. B complex	284.3	84.8
				Barley		
12	35	5	1.75	Pearled barley	262.1	24.1
13	25	4	0	” ”	412.8	42.2
14	14	2	1.75	Autocl. pearled barley	327.1	33.0
15	14	2	1.75	Malted barley	472.1	46.5
16	7	1	1.75	Pearled barley + Vit. B complex	328.6	70.2
				Misc.		
17	7	1	1.75	Wheat flour	382.9	69.1
18	7	1	1.75	Wheat germ	491.4	64.5
19	7	1	1.75	Oats	387.1	60.0
20	7	1	1.75	Corn meal	395.7	50.3
21	6	1	1.75	Soya bean flour	228.3	46.7
22	6	1	0	” ” ”	395.0	52.1
				Synthetic		
23	7	1	1.75	(a) Synthetic diet I	484.3	53.7
24	7	1	1.75	(b) ” ” I	475.7	73.6
25	7	1	1.75	” ” II†	555.7	83.4
26	7	1	1.75	” ” III†	486.0	25.9

TABLE III.
Effect on Tumor Growth of 4 Intravenous Injections of Various Extracts Given Over a Period of 48 Hours

No. of animals in group*	Type of extract	Dose, mg	Mean initial size, in^2	Standard error	Mean terminal size, in^2	Standard error	Mean terminal wt, mg	Standard error
7	Standard preparation	5.25	.171	.009	.167	.013	283	51
7	Polished rice	2.40	.179	.011	.171	.012	264	39
7	Pearled barley	3.60	.167	.010	.147	.014	200	38
7	” ”	1.20	.171	.010	.191	.021	310	60
7	Malted ”	7.50	.180	.004	.270	.008	686	39
7	Saline solution	0	.164	.007	.250	.011	627	75

* The animals were kept on a mouse pellet diet.

diet. Table III illustrates one such experiment. Whether this extractable tumor inhibitor is identical with the previously mentioned feeding factor, cannot be answered yet.

Similar feeding experiments were carried out with pearled barley, malted barley, wheat flour, wheat germ, oats, cornmeal and soya beans (see Table II). An increased inhibitory effect was obtained by feeding the animals pearled barley and soya beans (Table II, 12, 13, 14, 21, 22) while the effect of wheat flour, oats and cornmeal was less evident (Table II, 17, 19 and 20). No increased inhibiting effect was obtained by feeding malted barley and wheat germ (Table II, 15 and 18). Thus far, extracts were prepared only from pearled barley and malted barley. Intravenous injections of pearled barley of 0.1 cc corresponding to 1.2 mg solids showed an inhibitory effect similar to 5.25 mg of the standard when groups of animals were kept on normal diets. Extracts from malted barley were ineffective (Table III). At present no conclusions can be drawn as to the quantities of tumor growth inhibitors in the different grains. Experiments for the quantitative determination and chemical characterization of these factors are in progress.

Summary. Tumor growth-inhibiting substances are demonstrated in various grains with the aid of a rapid test on Sarcoma 180. They are effective by feeding in combination with injections of a standard solution of yeast extracts. Extracts of these grains are effective upon intravenous injection.

14117 P

Treatment of Spontaneous Breast Cancers in Mice with Pearled Barley.*

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We have presented in previous papers[1,2,3,4] our results with intravenous injections of yeast extract in the treatment of spontaneous breast adenocarcinomas in mice. We reported complete disappearance of these tumors in 30% of the animals, if the tumors were not of excessive size. In our last publication[4] we reported that by combining the injections of yeast extract with the feeding of polished rice we were able to double the percentage of complete regressions and to get these results not only in small tumors, but in tumors of considerable size.

We mentioned in this paper that since the preparation of beer had been changed, due to the exigencies of the war, successful results could be obtained only with a yeast extract prepared from bock beer, as bock beer was still prepared according to the old technic. Bock beer is prepared only once a year.

We were planning to prepare a large quantity of this extract when we were informed that the breweries had decided to discontinue the manufacture of bock beer for the duration of the war. Thus we were forced to abandon the use of yeast extract and to search for other sources of tumor growth-inhibiting substances.

In the preceding paper[5] we demonstrated on the growth-rate of a transplanted tumor (Sarcoma 180) an increased inhibitory effect, resulting from the combination of a polished rice diet and intravenous injections of yeast extract. From observations with other diets it was evident that this effect is not due to a deficiency in the diet. Furthermore it was possible to extract a factor from the rice which inhibited tumor growth when injected intravenously. As feeding experiments and injections of extract of pearled barley gave a similar inhibition of tumor growth in the rapid test, we tested the influence of pearled barley on spontaneous tumors.

In this preliminary report we shall present results obtained by treating mice with intravenous injections of pearled barley extract and a diet of pearled barley or polished rice.

The pearled barley extract is prepared in

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[1] Lewisohn, R., Leuchtenberger, C., Leuchtenberger, R., and Laszlo, D., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 558.

[2] Lewisohn, R., Leuchtenberger, C., Leuchtenberger, R., and Laszlo, D., *Am. J. Path.*, 1941, **17**, 251.

[3] Lewisohn, R., Leuchtenberger, C., Leuchtenberger, R., Laszlo, D., and Bloch, K., *Cancer Research*, 1941, **1**, 799.

[4] Lewisohn, R., Leuchtenberger, C., Leuchtenberger, R., Laszlo, D., and Dische, Z., *Cancer Research*, 1942, **2**, 218.

[5] Lewisohn, R., Laszlo, D., Leuchtenberger, C., Leuchtenberger, R., and Dische, Z., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 269.

the following manner:

To 100 g barley ("Albers Pearled Barley") 1500 cc distilled water plus 0.15 cc glacial acetic acid are added. The mixture is stirred, heated rapidly to 100° and boiled for 7 minutes. The supernatant fluid is separated by decantation from the barley, is cooled to room temperature and precipitated without concentration at 50% ethanol. It is kept in an ice-box overnight and filtered off. The filtrate is freed from ethanol in vacuum and concentrated to 30 cc. The turbid concentrate is filtered through a sterile Seitz-filter and poured into sterile ampoules. About 2.5% of the barley solids are extracted.

0.1 cc is injected intravenously every day, and injections must be continued until the tumors have disappeared completely.

The animals are fed pearl barley and carrots. After the tumors have disappeared, the mice are put back on the ordinary diet of pellets from the Rockland Farms and carrots.

We use in our work 3 different types of mice, the strain A and the Swiss strain from the Jackson Memorial Laboratory, and tumor mice obtained from the Rockland Farms.

Before the treatment is started the diagnosis of malignancy is established by a biopsy.

During the course of the treatment the average loss of weight of the animals was 5 g. However, the beneficial effect of this diet on tumor growth should not be ascribed to a deficiency in the diet.[5] Evidently the diet contains substances which inhibit tumor growth. Only the combination of the pearled barley diet with intravenous injections of barley extract effects a rapid disappearance even of large tumors in about one-half of the animals.

That diet alone is not sufficient to cause complete regression of tumor growth has been reported in a previous paper. Fifty controls received polished rice and carrots as a diet, but were not treated intravenously. In none of these animals did the tumors disappear. On the contrary, most of the animals showed a very rapid tumor growth.

The changes which occur in these tumors following this combined treatment are striking. After a few treatments hemorrhagic tumors show a whitish color. Hard tumors change to a soft consistency. In many cases the whole tumor is expelled, leaving a flat cavity which heals completely after a few days.

Detailed macroscopic and microscopic descriptions of these changes will be presented in a subsequent publication.

Among 46 spontaneous breast adenocarcinomas 19 tumors regressed completely.

At present we have a set of animals under observation in which pearled barley is replaced by brown barley, both in the diet and in the extracts for the intravenous therapy.

14118

Effect of Oil of Peppermint, Dehydrocholic Acid and Disodium Phosphate on Gastric Emptying in Dogs.

J. Clifford Stickney. (Introduced by Edward J. Van Liere.)

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The results reported here were obtained in an endeavor to find a simple substance which would reduce to normal the increased gastric emptying time produced by anoxia. Oil of peppermint,[1] dehydrocholic acid[2] and disodium phosphate[3] were chosen, because they have been reported to decrease gastric emptying time in human beings and the first named agent in dogs as well. In addition they have little or no complicating side effects.

Normal gastric emptying time was determined fluoroscopically on 4 dogs following a meal of 10 g ground dried bread, 20 g ground lean beef and 50 cc skim milk to which 15 g barium sulfate were added. From 6 to 11 control determinations were made on each dog to establish the mean. In the case of dogs 2, 3 and 4 the control and experimental values were obtained with the dogs in a low-pressure chamber at normal atmospheric pressure when the experiments with oil of peppermint were undertaken. For the other experiments the dogs were kept in their accustomed quarters. The dehydrocholic acid and the disodium phosphate were mixed with the test meal while the oil of peppermint was given orally in a capsule immediately before the test meal.

The dogs weighed from 8.6 to 9.8 kg. The effect of oil of peppermint was tested at three dosage levels: 0.1, 0.2 and 1.0 cc. Dog 4 vomited on two occasions with the 1.0 cc dose, so it was concluded that no larger amount could profitably be given. The amount of dehydrocholic acid administered was 0.5 g. Disodium phosphate was tested at the 0.5 and 1.0 g dosage levels. In every case with one exception four or more trials were made on each drug at each dosage level.

The results are given in the accompanying tables along with the calculation of Fisher's "p"[4] to determine the significance of the differences. It will be noticed that with 2 exceptions gastric emptying time was increased in every case when the groups are taken as a whole, but with certain dogs there was a significant increase for certain dosage levels of the drugs. Dog 1, which was also given oil of peppermint at the 0.2 cc dosage level, had a significant increase in gastric emptying time after 0.1 cc and 0.2 cc oil of peppermint.

TABLE I.
Effect of 0.1 and 1.0 cc Oil of Peppermint on Gastric Emptying Time.

Dog No.	Control, hr	After 0.1 cc oil of peppermint, hr	Difference, hr	Fisher's "p"	After 1.0 cc oil of peppermint, hr	Difference, hr	Fisher's "p"
1	5.50	6.30	+.80*	$<.001$	5.85	+ .35*	$>.05$
2	5.16	5.81	+.66	$>.10$	5.48	+ .32	$>.20$
3	5.78	6.75	+.97	$>.10$	7.06	+1.27*	$<.01$
4	4.83				4.94	+ .11	$>.20$
Avg	5.48†	6.29	+.81	$>.05$	5.83	+ .51	$>.20$

* Significant when "p" is .05 or less.
† Dog 4 not included.

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[1] Sapoznik, H. I., Arens, R. A., Meyer, J., and Necheles, H., *J. Am. Med. Assn.*, 1935, **104**, 1792.

[2] Van Liere, E. J., and Northup, D. W., *Am. J. Dig. Dis.*, 1941, **8**, 26.

[3] Van Liere, E. J., and Sleeth, C. K., *Am. J. Dig. Dis.*, 1940, **7**, 118.

[4] Fisher, R. A., *Statistical Methods for Research Workers*, fourth edition, Oliver and Boyd, London, 1932.

TABLE II.
Effect of 0.5 and 1.0 g Disodium Phosphate on Gastric Emptying Time.

Dog No.	Control, hr	After 0.5 g disodium phosphate, hr	Difference, hr	Fisher's "p"	After 1.0 g disodium phosphate, hr	Difference, hr	Fisher's "p"
1	5.50	5.79	+.29	>.20	5.80	+.30	>.20
2	5.32	5.85	+.53*	>.10	5.10	—.22	>.20
4	4.94	5.30	+.36	>.10	5.62	+.69*	>.05
Avg	5.25	5.64	+.39	>.20	5.51	+.26	>.20

* Significant when "p" is .05 or less.

TABLE III.
Effect of 0.5 g Dehydrocholic Acid on Gastric Emptying Time.

Dog No.	Control, hr	After dehydrocholic acid, hr	Difference, hr	Fisher's "p"
2	5.32	5.62	+ .31	>.20
3	5.40	5.00	— .40	>.20
4	4.94	6.06	+1.12*	<.01
Avg	5.22	5.56	+ .34	>.20

* Significant when "p" is .05 or less.

In the case of oil of peppermint, the results confirm the work of Van Liere and Northup,[5] who found no change in gastric emptying time in human subjects. The lack of confirmation of the work of Sapoznik *et al.*[1] may be attributed to their use of a different method to determine gastric emptying in their dogs and also to their use of very large doses of oil of peppermint. In regard to dehydrocholic acid and disodium phosphate it is difficult to explain why the dogs reacted differently than did the human subjects of Van Liere and his coworkers.[2,3]

Conclusions. The gastric emptying time of dogs has not been found to be significantly changed on the whole by the administration of oil of peppermint, dehydrocholic acid or disodium phosphate at the dosage levels used.

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[5] Van Liere, E. J., and Northup, D. W., *J. Pharm. and Exp. Ther.*, 1942, **76**, 39.

14119

Intestinal Flora of Rats on Purified Diets Containing Sulfonamides.*

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It has been established that there is a reduction in the growth rate of rats on synthetic diets when receiving certain sulfonamides. The phenomenon was first reported by Black *et al.*[1] in rats receiving a basal diet containing the 6 B vitamins, thiamine, pyridoxine, riboflavin, nicotinic acid, calcium pantothenate, and choline. The sulfanilylguanidine (sulfaguanidine) was added at a level of 0.5% in the ration. This observation has been confirmed by several workers.[2-8] Welch[9] first reported the antibacterial activity of succinylsulfathiazole (sulfasuxidine) and its relation to nutrition experiments. Similar results have been obtained by other workers upon administration of succinylsulfathiazole.[5-10]

There is considerable evidence that the growth of the rats on the synthetic diet is due at least in part to the intestinal synthesis of the additional vitamins. The work with sulfonamide-containing diets has led to the suggestion that biotin[8,10,12] and folic acid[8,10,12] are synthesized in the intestinal tract in appreciable quantities. In view of these findings it seemed of value to study the intestinal flora of rats under these conditions.

Rats from the Sprague-Dawley colony were chosen because of their freedom from protozoan and metazoan parasites, thus eliminating possible changes from such organisms. Weanling rats were fed the basal diet of Black *et al.*[1] with supplements as follows: Group I, basal diet only; Group II, basal diet plus a daily supplement of 0.4 g of solubilized liver;‡ Group III, basal diet plus 0.5% sulfaguanidine; Group IV, basal diet plus 0.5% sulfaguanidine and liver as above.

Each week a sample of 0.1 g of feces was weighed from the freshest specimens in a 12-hour collection and bacteriological observations were made. The sample was placed in a 10 cc water blank and dilutions were made from this emulsion. "Total" counts were made by inoculations from the different dilutions into a liver extract medium containing liver tissue. Eosin-methylene blue plates were

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† Eli Lilly & Co. Fellow.

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[8] Martin, Gustav J., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 353.

[9] Welch, A. D., *Federation Proc.,* Part II, Fed. Am. Soc. Exp. Biol., 1942, **1**, 171.

[10] Nielsen, Edward, and Elvehjem, C. A., *J. Biol. Chem.,* 1942, **145**, 713.

[11] Daft, Floyd S., Ashburn, L. L., and Sebrell, W. H., *Science,* 1942, **96**, 321.

[12] Mitchell, Herschel K., and Isbell, Edith R., *The University of Texas Publication,* No. 4237, 1942, 124.

‡ The solubilized liver is that fraction which is precipitated from an aqueous liver extract by the addition of ethyl alcohol to 70% concentration, then rendered water-soluble by enzymatic action.

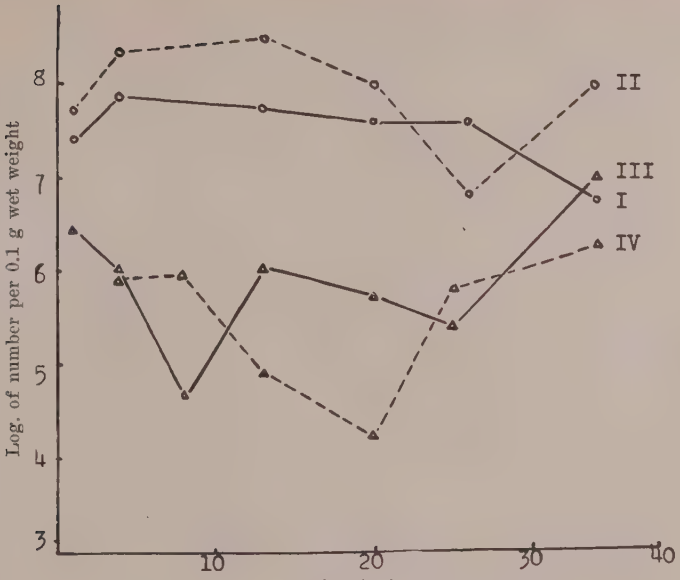

FIG. 1.

E. coli in feces as follows: I, basal diet only; II, basal plus liver extract; III, basal plus sulfaguanidine; IV, basal plus sulfaguanidine plus liver.

used for *E. coli* counts.

The most significant change noted in the feces was a drop in the number of *E. coli* which became evident as early as the third and fourth days after feeding the ration containing sulfaguanidine. The number remained low for 20-25 days but after that period there was a gradual return to normal. The results are shown in Fig. 1.

Succinylsulfathiazole appears to be equally as effective as sulfaguanidine in its antibacterial activity.[13] Also evidence has pointed to folic acid[10] as one of the unknown factors described by Black *et al.*[1] Thus a second experiment was carried out using succinylsulfathiazole with the following basal diet: sucrose, 76%; casein, 18%; salts, 4%; corn oil, 2%. The B vitamins were added at the following levels: pyridoxine, 250 γ; thiamine, 200 γ; riboflavin, 300 γ; nicotinic acid, 250 γ; calcium pantothenate, 2 mg; choline hydrochloride, 100 mg per 100 g of ration. Each rat was given 2 drops of a 1:6 dilution of haliver oil each week. Six groups of rats were run as follows: (a) basal diet only, (b) basal diet plus liver extract as in the first experiment, (c) basal diet plus a daily supplement of crude folic acid concentrate,[§] (d) basal diet plus 0.5% succinylsulfathiazole, (e) basal diet plus succinylsulfathiazole and liver as above, (f) basal diet plus succinylsulfathiazole and folic acid concentrate as above.

Because of a slight diarrhea resulting from the succinylsulfathiazole it was impractical to collect feces. Therefore, rats were sacrificed at various intervals through the 5-week period and the gastro-intestinal tract studied at 4 different levels, stomach, duodenum, cecum

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[13] Poth, Edgar J., Knotts, F. Louis, Lee, James T., and Iniu, Frank, *Arch. Sugg.*, 1942, **44**, 187.

[§] The method of Hutchings, Bohonos and Peterson[14] was used for the preparation of the folic acid concentrate. The amount of the concentrate given had the same folic acid activity as the 0.4 g solubilized liver given to group (b). The assay was made according to the method of Mitchell and Snell.[15]

[14] Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, 1941, **141**, 521.

[15] Mitchell, Herschel K., and Snell, Esmond E., *The University of Texas Publication*, No. 4137, 1941, 36.

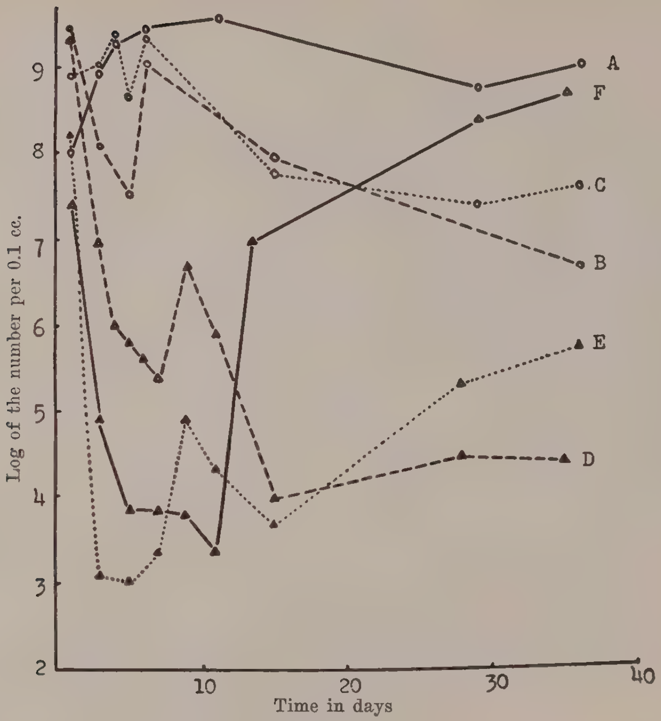

FIG. 2.

E. coli in cecal contents as follows: A, basal only; B, basal plus liver extract; C, basal plus folic acid concentrate; D, basal plus succinylsulfathiazole and liver; E, basal plus succinylsulfathiazole and folic acid concentrate; F, basal plus succinylsulfathiazole.

and colon. 0.1 cc samples of the contents of the specific level were measured with a blunt-end pipette and dilutions made as with the fecal samples.

The bacterial flora of the stomach varied since there was no starvation period before sampling and no real attempt was made to prevent coprophagy. When the stomach contents showed the presence of fecal material the total number increased and some *E. coli* were present. When the stomach was relatively empty the counts were low. Duodenal samples were quite constant except for the occurrence of *E. coli* when large numbers of that organism were found in the stomach. The total numbers were comparatively low. The bacterial picture was much the same in the cecum and colon. Because of the more uniform consistency of cecal contents and a greater possibility of accurate measurement of samples, the cecal counts only are reported here.

As shown in Fig. 2, there is a reduction in the number of *E. coli* as early as the third day after the experiment was begun with all rats receiving succinylsulfathiazole. When succinylsulfathiazole only was added to the basal diet, the count remained low for 11 days but showed a rather rapid increase during the next 5 days and was within the control range by the 4th week. Rats receiving the drug supplemented with the folic acid concentrate showed the greatest reduction of any of the groups. The return to normal was more gradual than in the unsupplemented group and at the end of 5 weeks had not reached the control range. The rats receiving the succinylsulfathiazole with liver extract showed a

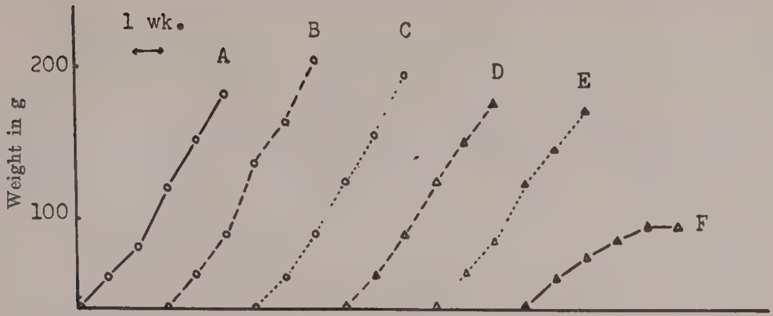

FIG. 3.
Growth curves for rats on diets as follows: A, basal only; B, basal plus liver extract; C, basal plus folic acid concentrate; D, basal plus succinylsulfathiazole and liver; E, basal plus succinylsulfathiazole and folic acid concentrate; F, basal plus succinylsulfathiazole.

more gradual decline in the number of *E. coli*, indicating the presence of something in the liver which counteracts to some extent the action of the drug. The count was still low at the end of 5 weeks.

The "total" number of organisms in the cecum remained constant, $1x10^{10}$-$1x10^{11}$ per cc, throughout the test period. As the coliforms decreased in number the enterococci increased thus keeping the total number the same. The large number of enterococci present in the feces of humans during succinylsulfathiazole administration was noted by Kirby.[16] Light and coworkers[6] reported no notable decrease in the total count, but an apparent difference in the composition of the flora in the feces of rats given sulfaguanidine.

All of the rats grew at a fairly normal rate except those receiving the synthetic diet plus succinylsulfathiazole with no supplement. The growth curves are shown in Fig. 3.

The results indicate that *E. coli* is the organism mainly responsible for the synthesis of biotin and folic acid or related factors. Thompson[17] reports the synthesis of biotin and folic acid by the closely related organism, *A. aerogenes*. Evidently resistant strains of *E. coli* adapt themselves to the presence of the drug[6,18] and the number returns to normal. However, in spite of the fact that the number of organisms as measured in this study increase, the ability of the organism to synthesize the important growth factors must have been lost since the animals show no growth during the period that the number of organisms increase. The greater repression of coliforms with succinylsulfathiazole than with sulfaguanidine is in agreement with Poth *et al.*[13] The reduction in the number of *E. coli* is more complete with the crude diet (basal + succinylsulfathiazole + liver) than on the purified diet (basal + succinylsulfathiazole), which is probably the reason Poth and his coworkers did not encounter a return of coliform counts during succinylsulfathiazole administration with dogs on a meat diet.

Summary. Rats receiving purified diets plus sulfaguanidine and succinylsulfathiazole showed a decrease in the number of *E. coli* in the feces and cecum within three days after the drug was given. The number remained low for 10 days and then began to increase and at the end of the five weeks approached the normal level.

The "total" counts remained constant throughout the test period. Enterococci replaced the coliforms during the time of repressed *E. coli* growth.

On a succinylsulfathiazole-containing diet supplemented with either liver extract or a crude folic acid concentrate normal growth was maintained in spite of a marked reduction in the number of *E. coli* in the cecum.

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[16] Kirby, Wm., Rantz, M. M., and Lowell, A., *J. A. M. A.*, 1942, **119**, 615.

[17] Thompson, Roy C., *The University of Texas Publication*, No. 4237, 1942, 87.

[18] Vivino, Jean Jermsta, and Spink, Wesley W., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 336.

14120

Further Investigations on Presence of Rh Antibodies in Breast Milk.

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The occurrence of Rh antibodies in the breast milk of an Rh negative mother who had given birth to a child suffering from erythroblastosis was reported in a previous communication.[1] However, the first specimen of breast milk examined was taken at the beginning of the second week following delivery, which might have accounted for the low antibody titer. The possibility that Rh antibodies in breast milk could constitute a contributing factor in bringing about destruction of the child's blood cells was entertained. This warranted further studies on the occurrence of Rh antibodies in breast milk. Since then, we have had an opportunity to examine 2 more cases in which the Rh antibody was of a much higher titer than in the one previously described.

Case No. 1: A colored patient, aged 20, gave birth to a child who died 3 days after delivery with the clinical diagnosis of erythroblastosis confirmed at autopsy. The patient has 2 living children, one child who died at the age of 4 months, and one miscarriage. There was some doubt regarding the paternity of the different children. The mother, baby and putative father belonged to the blood group A. The mother was found to be Rh negative, the baby and father Rh positive. The mother's serum contained a potent Rh antibody and agglutinated the baby's cells in dilutions up to 1:256. A specimen of breast milk was obtained on the fifth day following delivery (two days after the baby's death). The technic used for the determination of the Rh antibodies was the same as that used for the determination of the Rh antibodies in blood serum; the milk specimen was centrifuged and the layer of fat removed with a swab. The turbid supernatant fluid was used for examination. Decreasing amounts of breast milk, volume .1 cc, were mixed with .1 cc each of a 1% suspension of Rh negative blood cells belonging to group O. The mixtures were incubated for 1 hr at 37°C and then centrifuged. The degree of agglutination observed was recorded following centrifugation. In dilutions up to 1:4 the resulting agglutination was very strong (4+ in character) but definite agglutination (1+) of Rh positive cells was obtained in dilutions of the breast milk up to 1:16. When tested against a number of Rh positive and Rh negative cells it was found that Rh positive cells were strongly agglutinated while Rh negative cells failed to show any agglutination.

Case No. 2: Blood specimens of parents and baby were sent to this laboratory from another hospital a few hours following delivery of the child. The child exhibited clinical symptoms of erythroblastosis. All 3 blood specimens belonged to the blood group O. The mother was Rh negative and the father and baby were Rh positive. The mother's serum agglutinated Rh positive cells up to a dilution of 1:8 though the degree of this agglutination was not too marked (2+). Colostrum was obtained about 24 hours following delivery. In spite of the fact that the Rh antibody content of the serum was not very high, examination of the colostrum revealed the presence of an Rh antibody of about the same strength as that observed in the blood serum. The colostrum specimen agglutinated Rh positive cells in a dilution up to 1:8.

Summary and Conclusions. Rh antibodies were found in the breast milk of 2 mothers with erythroblastotic children. The Rh antibody titer was much higher in both instances than in the case reported previously. In the first case, the Rh antibody titer of the serum was 1:256 while the milk specimen obtained on the fifth day following delivery showed an antibody titer of 1:16. In the second case, the Rh antibody titer of the serum was not higher than 1:8 and the colostrum, upon examination, revealed the same titer. Those findings suggest that the content of Rh antibodies in breast milk is relatively high in the first days following delivery, especially in the colostrum. In the few cases we examined, we have not been able to demonstrate antibodies in the breast milk unless they were present in the serum. Assuming that Rh antibodies in the breast milk pass through the intestinal canal and enter the circulation, breast milk might be the cause of further damage to blood cells of erythroblastotic children.

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14121 P

Inherited Differences in the Choline Requirement of Rats.*

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In a previous report[1] it was emphasized that there was considerable variation in the minimum choline requirement of rats from different litters. An experiment was conducted to determine whether or not this variation was hereditary. Two female and 2 male rats which had produced offspring having a relatively high choline requirement were mated, and their progeny were designated the F_1 of strain HCR. Likewise a similar number of rats which had produced offspring having a relatively low choline requirement were mated and their offspring were designated the F_1 of strain LCR. The F_1 rats of both strains were brother-sister mated simultaneously so that seasonal and other environmental influences would be uniform in the 2 strains.

At 23 days of age the F_2 rats were placed on the choline-deficient diet 31PMC[2] supplemented with adequate amounts of thiamine, riboflavin, pyridoxin, Ca pantothenate, carotene and calciferol. A suboptimum level of 4 mg of choline chloride per rat daily was also fed. Seventy-six progeny from 4 F_1 females of the strain HCR and 126 progeny from 6 F_1 females of strain LCR were tested over a period of 6 months. Weight gains of the progeny were equal in the 2 strains and ranged from 15 to 20 g per rat during the first week. Kidney hemorrhage, characteristic of choline-deficiency, developed in 6 to 10 days, and the severe cases culminated in death. The kidneys of survivors were examined at the end of a 2-week feeding period. The presence and severity of kidney hemorrhage were used as an index to the adequacy or inadequacy of choline nutrition.

The results are presented in Table I. With a daily intake of 4 mg of choline chloride, 100% of the F_2 males of strain HCR developed kidney hemorrhage, and 58% of them died. The incidence of hemorrhage among the F_2 males of strain LCR receiving the same level of choline was only 48%, with a mortality of only 10%. As previously reported, the females required less choline than the males, but again the incidence of hemorrhage was markedly higher in the F_2 females of strain HCR. All the F_1 females of strain HCR produced young about equal in their choline requirement, and similarly the offspring of the F_1 females in strain LCR, regardless of litter were uniform in their requirement of choline.

Preliminary results indicate that the daily requirement for choline is nearly twice as great in strain HCR as in strain LCR. That such unusual differences in the requirement for a specific nutrient can be demonstrated in the second generation seems highly significant, and illustrates clearly how genetic variation in the stock used may affect the results of nutrition experiments. The physiological basis for this genetic difference in the requirement of choline is yet unknown.

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* Published with the approval of the director of the Alabama Agricultural Experiment Station.

[1] Engel, R. W., Proc. Soc. Exp. Biol. and Med., 1942, **50**, 193.

[2] Engel, R. W., and Salmon, W. D., *J. Nutrition*, 1941, **22**, 109.

TABLE I.
Strain Differences in Choline Requirements.

Sex	Strain	F_2 rats, No.	Incidence of kidney hemorrhage No.	Incidence of kidney hemorrhage %	Mortality No.	Mortality %
Male	LCR*	60	29	48.3	6	10.0
Male	HCR†	43	43	100.0	25	58.1
Female	LCR	66	13	19.7	1	1.5
Female	HCR	33	31	93.9	14	42.4

* Low choline requirement strain.
† High choline requirement strain.

Griffith[3] has shown that the choline requirement increases with increased rate of growth, which is not an explanation for the present results since the growth rate was identical in the 2 strains.

Lamoreux and Hutt[4] found that white Leghorn chicks survived longer on a thiamine-deficient diet than Rhode Island Reds. The same authors[5] developed strains of White Leghorn chicks which appeared to have different requirements for riboflavin.

So far as the author is aware, apart from a report by Light and Cracas[6] who observed differences in thiamine requirements in different strains of white rats, it has not previously been demonstrated that laboratory rats vary in their requirements for specific nutrients.

Summary. Two strains of rats were developed which in their second generation differed markedly in their requirement of dietary choline.

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[3] Griffith, W. H., *Biol. Symposia*, 1941, **5**, 193.

[4] Lamoreux, W. F., and Hutt, F. B., *J. Agr. Res.*, 1939, **58**, 307.

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[6] Light, R. F., and Cracas, L. J., *Science*, 1938, **87**, 90.

14122

Cathartic Salts on Motility of Thiry-Vella Jejunal Loops.

E. WILLIAM LIGON, JR. (Introduced by George B. Roth.)

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Intralumen pressures in, and flow through, Thiry-Vella jejunal loops of 3 trained unanesthetized dogs were recorded photokymographically by the open tube method of Brody, Werle, Meschan and Quigley.[1] The solutions studied were: water; isotonic solutions of NaCl, Na_2SO_4, and $MgSO_4$, respectively; hypertonic solutions of 5 times the isotonic concentration of the above salts. Apparatus used is shown in Fig. 1.

Effect of pressure. Perfusion of the jejunal loop with isotonic (0.9%) NaCl at an inflow pressure of 20 cm of water and an outflow pressure of zero produced basal pressures around 10 cm, average peak pressure around 40 cm, flow about 10 cc/min, and contractions 12 to 15/min. On lowering inflow pressure to zero, basal pressure fell to about 3 cm, average peak fell to about 10 cm, and flow ceased. On raising inflow pressure to 40 cm, basal pressure rose to 20 cm, average peak rose to 75 cm, and flow doubled. On clamping inflow and outflow and introducing 10 cc of 0.9% NaCl, basal pressure rose to 70 cm and average peak to 100 cm. Rate of contraction remained

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[1] Brody, Werle, Meschan and Quigley, *Am. J. Physiol.*, 1940, **130**, 791.

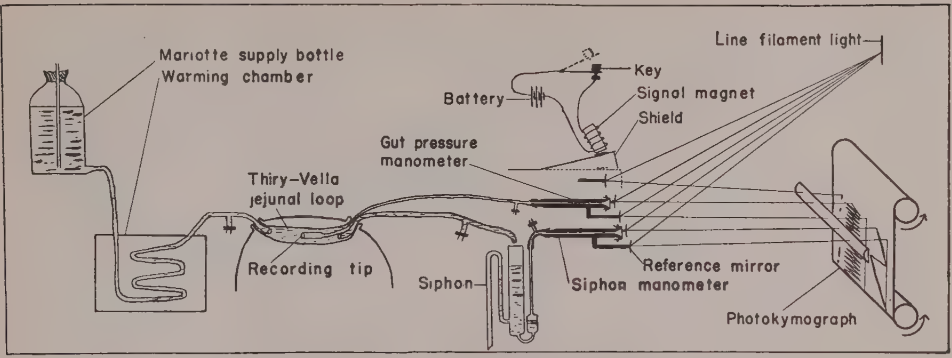

FIG. 1.
Diagrammatic representation of apparatus. The manometers are drawn disproportionately large.

unchanged throughout. Since motility of the jejunum varies directly with inflow pressure and an inflow pressure of 20 cm of water was required to cause a moderate flow, this pressure was routinely employed, with 0.9% NaCl as the control solution.

Effect of isotonic solutions (1.89% Na_2SO_4, 3.28% $MgSO_4$). Isotonic Na_2SO_4 usually caused a slight increase (15 to 25%) in average peak pressure and flow without change in rate (Fig. 2, A). However, if the control period was one of low motility and rapid flow, isotonic Na_2SO_4 caused a change toward the usual normal. The effects developed quickly and were maintained; return to isotonic NaCl brought return to previous levels of activity. Isotonic $MgSO_4$ usually caused about the same increase in motility as isotonic Na_2SO_4; when prolonged over 30 to 40 minutes, motility and basal pressures fell markedly and flow greatly increased.

Effect of anisotonic solutions (H_2O, 4.5% NaCl, 9.45% Na_2SO_4, 16.4% $MgSO_4$). Distilled water showed little effect. Once, there was depression of motility and increased flow for 13 minutes followed by a two-minute period of spasticity, absence of flow, and symptoms of nausea.

Hypertonic solutions caused a marked decrease in motility with greatly increased flow in 5 to 15 minutes. Usually there was a development of a spastic period in the first 5 minutes, sometimes followed later by other spastic periods. During these spastic periods (Fig. 2, B and C) the contractions continued at the usual rate, but pressures were quite high, peaks often rising well over 120 cm water pressure, with incomplete relaxation so that basal pressures frequently remained over 30 cm. Associated with these spastic periods were symptoms of nausea—licking of chops, swallowing; often abdominal gurgling, stretching movements, increased respiration. Flow was always increased during low motility and decreased during spastic periods. Rate of contraction was constant.

With hypertonic NaCl there was usually one early spastic period; with hypertonic Na_2SO_4 often several within 20 minutes; with hypertonic $MgSO_4$ spastic periods were sometimes absent. On changing from hypertonic NaCl or Na_2SO_4 to isotonic NaCl recovery was obtained in 5 to 15 minutes, but after hypertonic $MgSO_4$ recovery never occurred in 15 minutes and often was incomplete at 45 minutes.

Discussion. From these experiments, in which the factor of distension was largely eliminated, it would seem that the stimulant effects of isotonic Na_2SO_4 and isotonic $MgSO_4$ in the jejunum would be insufficient to alone produce the vigorous catharsis which usually ensues from the oral administration of these salts; coupled with the retention of water in the lumen by these difficultly absorbable salts, the stimulant effect probably increases the rate of passage of material through the jejunum. The late depression of motility by isotonic $MgSO_4$ and the slow recovery after hypertonic $MgSO_4$ indicate that if catharsis

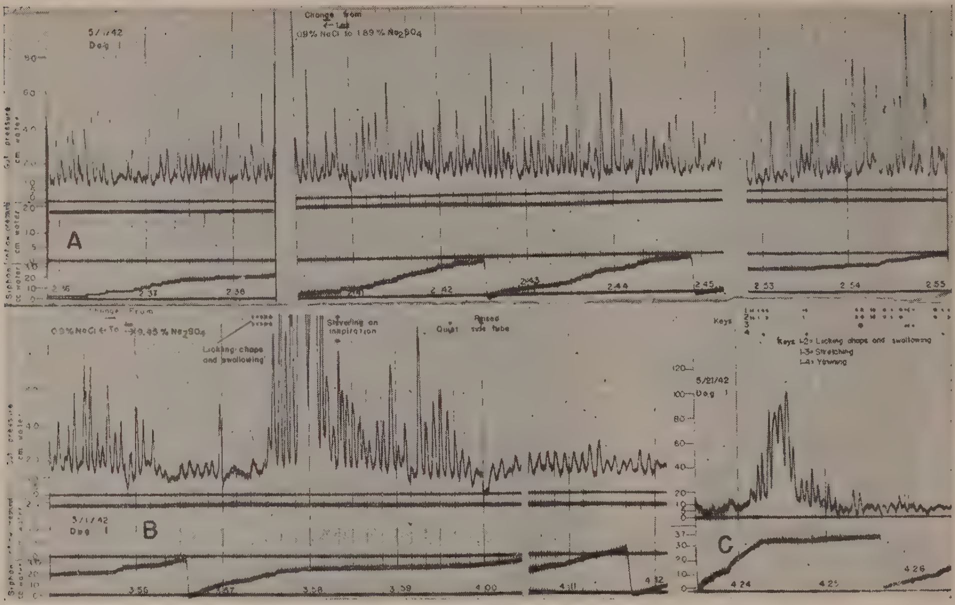

FIG. 2.

A. Typical record showing the moderate stimulation obtained with isotonic Na_2SO_4. B. Typical record showing an early spastic period at 3:58 and depression of motility at 4:11, using hypertonic Na_2SO_4. C. Typical record showing a late spastic period. Note that flow was very low during the spastic period.

failed to occur after oral administration, stasis of the solution in the jejunum would add to the danger of absorption of toxic amounts of the magnesium ion. Hypertonic solutions of these salines, should they reach the jejunum, would tend to remain stationary during periods of low motility; during spastic periods, material distal to the point of action would be driven faster and material proximal to this point would be held back.

Summary. 1. Isotonic Na_2SO_4 and isotonic $MgSO_4$ caused moderate stimulation of motility in Thiry-Vella jejunal loops; when prolonged, isotonic $MgSO_4$ caused depression of motility and tone. 2. Hypertonic solutions of NaCl, Na_2SO_4 or $MgSO_4$ caused depression of motility, with short periods of spasticity accompanied by symptoms of nausea. 3. Recovery after hypertonic NaCl or Na_2SO_4 was prompt, but after depression of motility by $MgSO_4$ recovery was slow. 4. Using a constant inflow pressure, flow bore an inverse relationship to motility. 5. Rate of contraction was unaffected by the agents employed.

14123

Effect of Cod Liver Oil and Rancidity on Certain Vitamin E Deficiency Symptoms.*

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It is known that cod liver oil added to a vitamin E deficient diet will accelerate the development of such symptoms as the muscular dystrophy in rabbit and guinea pigs,[1] whereas hydrogenated cod liver oil has no such effect.[2] Earlier suggestions that the effect of cod liver oil might be due to a destruction of traces of vitamin E in the diet through rancidity of the unsaturated fatty acids, either before or after their ingestion, have received added support by the recent studies of Mattill and Golumbic[3] who found that rancidity of the fat aggravated the symptoms. It would obviously be of interest to investigate how far the same considerations might be applied to another manifestation of vitamin E deficiency, namely, the increased capillary permeability in chicks.

Earlier studies[4] have already shown that lard and cod liver oil given in sufficient amount will accelerate the appearance of both exudates and encephalomalacia. These studies have since been extended to comprise an investigation of the influence of rancidity as well as that of certain fatty acids on the symptoms. It has thereby been possible to draw certain conclusions as to the role played by fats.

Procedure. The vitamin E deficient diet to which the various fats were added consisted of: alcohol-extracted casein 15 g, McCollum's salt mixture No. 185 supplemented with Cu and Mn 7.2 g, ether-extracted dried yeast 10 g, gelatin 8 g, gum arabic 5 g, choline chloride 0.1 g, cystine 0.1 g, sucrose 54.6 g, diacetate of 2-methyl-1,4-naphthohydroquinone 1 mg. The animals received, twice a week, one drop of a concentrate of vitamins A and D dissolved in cod liver oil (28.6 of each 29 mg drop consisted of cod liver oil) during the first 5 weeks, providing 48 I.U. of A and 5 chick units of D per day. This diet is not complete for chicks but adequate for the purposes of these experiments. With the exception of fats which were to be made rancid in the diet, all fats were added to the base every day. The chicks were day-old White Leghorns.

Results. The data presented in Table I demonstrate that fresh cod liver oil is extremely effective in accelerating the capillary symptoms; whereas cod liver oil which has been made thoroughly rancid, either before or after it is mixed with the diet, has no such effect.

It may therefore be concluded that cod liver oil has an effect on the capillary symptoms which is not limited to a destruction through rancidity of possible traces of vitamin E in the diet, or to the presence of rancidity products in the diet. A rancidity product, heptaldehyde, tested directly showed no effect. Furthermore, since addition of fresh cod liver oil to a diet containing rancid cod liver oil will also accelerate the symptoms, it may be assumed that rancidity does not result in the formation of a factor which protects against the symptoms.

Cod liver oil and lard are more effective in accelerating the capillary symptoms than fatty acids from linseed oil, while oleic acid is ineffective (Table II). This indicates that the effect is due to the presence of highly

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* Aided by a grant from the Josiah Macy, Jr., Foundation.

[1] Madsen, L. L., McCay, C. M., and Maynard, L. A., *Cornell Univ. Agric. Exp. Sta. Memoir*, No. 178, 1935.

[2] McCay, C. M., Paul, H., and Maynard, L. A., *J. Nutr.*, 1938, **15**, 367.

[3] Mattill, H. A., and Golumbic, C., *J. Nutr.*, 1942, **23**, 625.

[4] Dam, H., Glavind, J., Prange, I., and Ottesen, J., *Kgl. Danske Videnskabernes Selskab, Biologiske Meddelelser* (Royal Danish Academy of Science, Biological Communications), 1941, **16**, 7.

TABLE I.
Influence of Fresh and Rancid Cod Liver Oil on Exudate and Encephalomalacia in Chicks.

Additions to the basal diet	No. of birds with symptoms within 28 days		No. of birds without symptoms within 28 days
	Exudate	Encephalomalacia	
1. None	0	0	10
2. 5% fresh cod liver oil (iodine value 173)	8	1	1
3. 5% cod liver oil, made rancid[*] and added to diet (iodine value 86)[†]	0	0	9
4. 5% cod liver oil, made rancid in diet[‡] (iodine value 62)	0	0	9
5. As No. 4 + an additional 5% of fresh cod liver oil	8	0	2
6. 5% cod liver oil made slightly rancid[§] before adding to diet (iodine value 149)	9	0	1
7. 0.5% heptaldehyde[‖]	0	0	7

[*] By rapidly aerating at 100°C for one week.

[†] The decrease in iodine value (Rosenmund-Kuhnhenn method) gives some indication of the degree of rancidity.

[‡] By standing on shallow trays at 50°C for 1 week.

[§] By slowly aerating at 100°C for 24 hours.

[‖] The heptaldehyde was mixed with the food several times during the day, because of the volatility of the compound.

TABLE II.
Influence of Different Fats and Fatty Acids on Exudate and Encephalomalacia in Chicks.

Additions to basal diet	No. of birds with symptoms within 35 days		No. of birds without symptoms within 35 days
	Exudate	Encephalomalacia	
1. None	0	0	10
2. 4.8% oleic acid	0	0	10
3. 4.8% fatty acids from linseed oil	4	0	6
4. 5% lard	6	2	2
5. 5% cod liver oil	8	2	0

unsaturated fatty acids. The nature of this accelerating effect is under investigation at present.

The question arises as to whether exudates or encephalomalacia can be produced by diets absolutely free from highly unsaturated fat. In the experiments of Dam, Glavind, Prange and Ottesen[4] a few such symptoms were observed when no fat was added to the diet, but the latter was not so thoroughly extracted as that used in the present experiments. The fact that no capillary symptoms appeared in chicks reared on the basal diet used in the present studies, even after period longer than the 5 weeks indicated in Table II, suggests the importance of dietary fat in the genesis of the symptoms characteristic of vitamin E deficiency in the chick. The fact that the rancid state of fats does not accentuate the capillary symptoms in chicks, but appears to have a marked effect on muscular lesions in rabbits,[3] suggests that the way in which fats influence these types of lesions may be different. This, in turn, suggests the need for information concerning the effect of fatty acids and their rancidity products on muscular disturbances in the chick and related species.

Summary. The exudative diathesis, characteristic of vitamin E deficiency in chicks, readily occurs with diets containing 5% of fresh cod liver oil, slightly rancid cod liver oil, or a mixture of fresh and completely rancid cod liver oil; and to a lesser extent by similar proportions of lard or fatty acids from linseed oil. On the other hand, the symptoms do not occur if the diet contains a similar proportion of oleic acid or thoroughly rancid cod liver oil, or if the diet is rigidly freed of fats. These observations indicate that the appearance of symptoms of increased capillary permeability is dependent upon the presence of highly unsaturated fatty acids in the vitamin E deficient diet, and that rancidity of the dietary fat does not contribute to the development of the symptoms.

14124

Use of Thioglycollate Media for Testing Disinfectants.*

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The results which we obtained with an *in vivo* "infection-prevention" method for evaluating skin disinfectants[1] indicated that the mercurial compounds tested were actually quite ineffective, despite their accepted high disinfecting power as determined by the standard *in vitro* methods. The conclusion was drawn that failure of growth in the test tube probably reflected only bacteriostatic action, and that dilution of the mercurial or its combination with substances present in circulating blood or lymph might free the bacteria of the mercurial and result in infection and death of a test animal.

Brewer has suggested that the addition of sodium thioglycollate to media interferes with the bacteriostatic action of mercurials.[2] Acting on this suggestion, the National Institute of Health has recommended that this substance be added to media used for testing the sterility of biologicals containing mercurial preservatives.

On this basis, it was decided to determine whether the addition of sodium thioglycollate to media used in testing disinfectants by an *in vitro* method would give results similar to those obtained by the "infection-prevention" method.

The mercurial compounds used in the experiments to be discussed included mercuric chloride, mercuric oxycyanide, and aqueous solutions of metaphen, merthiolate, mercresin, and mercurochrome; the non-mercurial disinfectants were phenol, Lugol's iodine, and aqueous phemerol. A loopful of a broth culture of *Staphylococcus aureus* was placed in 5 ml of various concentrations of each of the disinfectants. After 10 minutes, these solutions were subcultured both into extract broth, and into extract broth containing 0.1% sodium thioglycollate.

The results summarized in Table I indicate that, with the non-mercurial disinfectants, essentially the same results were obtained whether or not thioglycollate was present in the medium. However, without exception, in the case of the mercurial disinfectants, the percent of positive growths was much greater when subcultures were made to the thioglycollate-containing media.

In the first experiments, the 0.1% concentration of thioglycollate was arbitrarily selected. However, the optimum concentration for inhibiting the bacteriostatic effect of the mercurial compounds was later determined to be between 0.05 and 0.10% (Table II). A greater concentration, 0.20%, retarded the growth of the test organism.

In further experiments, sodium thioglycollate was added to tubes of plain broth to give 0.1% concentration at intervals after they had been inoculated with staphylococci previously exposed to the various disinfectants for 10 minutes. It will be noted in Table III, which gives representative results of such

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* Aided by a grant from Parke, Davis and Company.

[1] Nungester, W. J., and Kempf, Alice H., *J. Infect. Dis.*, 1942, **71**, 174.

[2] Brewer, John H., *J. A. M. A.*, 1939, **112**, 209.

TABLE I.

Comparison of Plain and Thioglycollate-Containing Media for Detecting Surviving Bacteria in Testing Disinfectants.

			% of tests with positive growth	
Disinfectant*	Concentration	No. of tests	Plain broth	Broth containing sodium thioglycollate
Lugol's Iodine	1:100	2	0	0
	1:200	5	60	60
	1:500	7	72	72
	1:1000	8	87	75
Phemerol	1:2500	4	25	10
	1:5000	10	40	50
	1:10,000	7	72	86
	1:20,000	4	100	100
Phenol	1:70	10	10	10
	1:80	9	22	22
	1:90	9	78	78
Mercuric Chloride	1:5000	8	0	88
	1:10,000	7	0	86
	1:20,000	7	43	86
	1:50,000	5	40	80
Metaphen	1:500	2	0	100
	1:1000	9	0	67
	1:5000	10	20	90
	1:10,000	7	43	86
Merthiolate	1:1000	10	20	100
	1:5000	10	60	100
	1:10,000	10	70	100
Mecresin	1:500	4	25	0
	1:25,000	5	40	60
	1:50,000	6	67	100
Mercuric Oxycyanide	1:250	6	17	83
	1:1250	3	67	100
	1:2500	5	80	100
Mercurochrome	1:50	8	50	75
	1:250	7	57	100
	1:500	6	67	100

* All the disinfectants were aqueous solutions.

experiments, that growth was obtained when as long as 5 hours had elapsed between subculture of staphylococci previously exposed to mercuric chloride and the addition of sodium thioglycollate, while tubes with no thioglycollate did not show growth. After eight hours, however, exposure to the mercuric compound apparently produced death of the organism, since the staphylococcus culture survived comparable dilution in 0.85% sodium chloride solution for a time interval equal to the duration of the experiment. Similar results were obtained with the other mercurial disinfectants tested.

Summary. Sodium thioglycollate added to media for testing disinfectants *in vitro* apparently neutralized the bacteriostatic effect of the mercurial compounds tested and thus indicated the inadequacy of these substances as germicides.

TABLE II.
Effect of Various Concentrations of Sodium Thioglycollate in Media for Detecting Surviving Bacteria in Testing Disinfectants.

		% of thioglycollate in medium											
		0.00		0.01		0.02		0.05		0.10		0.20	
Disinfectant	Concentration	hrs: 24	48	24	48	24	48	24	48	24	48	24	48
Phemerol	1:50,000	—	+	—	—	—	—	—	—	—	+	—	+
	1:100,000	+	+	+	+	+	+	+	+	—	+	—	—
	1:250,000	+	+	+	+	+	+	+	+	+	+	+	+
Phenol	1:70	—	—	—	—	—	—	—	—	—	—	—	—
	1:80	—	—	—	—	—	—	—	—	—	—	—	—
	1:90	—	—	—	—	+	+	—	+	—	+	—	+
Mercuric Chloride	1:5000	—	—	—	—	—	—	+	+	+	+	—	—
	1:10,000	—	—	—	—	—	+	+	+	+	+	—	+
	1:50,000	—	—	—	—	—	+	+	+	+	+	—	+
Metaphen	1:500	—	—	—	—	—	+	+	+	—	+	—	+
	1:1000	—	—	—	—	—	+	+	+	+	+	—	+
	1:5000	—	—	—	—	—	+	+	+	+	+	—	+
Merthiolate	1:1000	—	—	—	—	—	—	+	+	+	+	—	+
	1:5000	—	—	—	—	+	+	+	+	+	+	—	+
	1:10,000	—	—	—	+	+	+	+	+	+	+	—	+
Mercurochrome	1:50	—	—	—	—	+	+	+	+	—	+	—	+
	1:250	—	—	—	—	+	+	+	+	—	+	—	+
	1:500	—	—	—	+	+	+	+	+	+	+	—	+

(+) indicates growth; (—) indicates no growth.

TABLE III.
Effect of Addition of Sodium Thioglycollate to Plain Broth at Intervals After Subculturing from Mercurial Solutions.

		Interval after subculturing									
		Min					Hr				
Disinfectant	Control	1	2	5	10	30	1	2	5	8	15
Mercuric Chloride, 1:5000	—	+	+	+	+	+	+	+	+	—	—
Metaphen, 1:500	—	+	+	+	+	+	+	+	—	—	—
Merthiolate, 1:1000	—		+				+		+	+	—
Mercuric Oxycyanide, 1:250	—	+	+	+	+	+	+	+	—	—	—
Mercurochrome, 1:50	—	+	+	+	+	+	+	+	—	—	—

14125

Antigenic Relationship Between *H. Influenzae* Type B and Pneumococcus Type VI.

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Recently, two reports were published pertaining to antigenic relationships between *Hemophilus influenzæ* and pneumococcus. Chapman and Osborne[1] isolated a strain of *H. influenzæ* type a from the spinal fluid and nasal secretion of a child suffering from a fractured skull. This particular strain gave a capsular swelling reaction with anti-pneumococcus type 6b serum. A strain of *H. influenzæ* type c, recovered from the sputum of a patient with upper respiratory infection, reacted with antipneumococcus type 21 serum. Neter[2] found that certain anti-*H. influenzæ* horse sera reacted with pneumococcus type 6, causing both precipitation and agglutination. It was reported, furthermore, that anti-*H. influenzæ* types a and b rabbit sera did not contain antibodies against pneumococcus type 6, nor did anti-pneumococcus type 6 horse serum react with *H. influenzæ* type b. In order to determine the nature of the type 6 pneumococcal antibodies in anti-*H. influenzæ* horse sera and the possible antigenic relationship of the two microörganisms involved, precipitation and cross-absorption experiments with the specific soluble substance of pneumococcus type 6 and the specific polysaccharide of *H. influenzæ* type b, were carried out. The results of this investigation are herewith presented.

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[1] Chapman, O. D., and Osborne, W., *J. Bact.*, 1942, **44**, 620.

Material and Methods. Several lots of anti-*H. influenzæ* horse sera were obtained through the kindness of Dr. Leah Seidman, Department of Bacteriology and Immunology, Harvard Medical School, Boston, Massachusetts, and Dr. Morris Shaffer, Massachusetts State Antitoxin Laboratory. Dr. H. E. Alexander kindly supplied *H. influenzæ* type b polysaccharide; 2 cc of the material contained 1.96 mg of the substance. Each milligram of this polysaccharide, according to Dr. Alexander,[3] absorbs 6.7 mg of type b *H. influenzæ* antibody nitrogen. Dr. Edwin F. Voight, Lederle Laboratories, furnished 100 mg of pneumococcus type 6 specific soluble substance (No. T6-38).

Precipitation tests were carried out in test tubes (3″x⅜″). The material was measured by means of Kahn pipettes. Precipitation was read macroscopically.

Results. Several lots of anti-*H. influenzæ* horse sera were tested for the presence of antibodies against pneumococcus type 6, as reported previously.[2] Serum No. I 60 markedly reacted with pneumococcus type 6. The titer of type 6 pneumococcal antibodies of several other lots of serum had definitely decreased during storage at icebox temperature for 5 months. The experiments with the specific soluble substance of pneumococcus type 6 and the polysaccharide of *H. influenzæ* were carried out with the serum No. I 60.

A titration of the anti-*H. influenzæ* serum (No. I 60) revealed that, with the supernatant fluid of a pneumococcus type 6 brain heart infusion broth culture as antigen, it caused precipitation in dilutions of 1:1 and 1:2; in dilutions of 1:4 and higher no definite reaction took place. For this reason, all experiments were carried out with undiluted serum.

In the first experiment it was determined whether or not this anti-*H. influenzæ* serum reacts with SSS of pneumococcus type 6. For comparative purposes an anti-pneumococcus type 6 horse serum was included. Various amounts of SSS, ranging from 2 mg to 0.00002 mg (volume 0.1 ml), were mixed with 0.05 ml of the undiluted sera. The results of this experiment are presented in Table I.

It may be seen from Table I that both anti-*H. influenzæ* serum and anti-pneumococcus type 6 serum reacted strongly with SSS of pneumococcus type 6. With both sera a zone-phenomenon was encountered. There cannot be any doubt, therefore, that the reactivity of anti-*H. influenzæ* horse serum with pneumococcus type 6 is due to the presence of antibodies directed against the specific soluble substance of this microörganism.

In order to ascertain the antigenic relationship, if any, between *H. influenzæ* type b and pneumococcus type 6, the following cross-absorption tests were carried out: To 0.6 ml of anti-*H. influenzæ* serum was added 0.025 ml of 1:10 diluted specific soluble substance of pneumococcus type 6 in physiological saline solution (0.05 mg). Precipitation occurred almost immediately. The mixture was incubated at 37°C for 2 hours and then centrifuged. The supernatant fluid, which was perfectly clear, was tested for the presence of antibodies reacting with the specific polysaccharide of *H. influenzæ* type b and also with SSS of pneumococcus type 6. In order to detect small quantities of antibodies the tests were carried out with decreasing amounts of the specific polysaccharide. For comparative purposes precipitation tests were included

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[2] Neter, E., *J. Immunol.*, 1943, **46**, 239.

[3] Alexander, H. E., personal communication.

TABLE I.
Reaction Between Anti-*H. influenzæ* (Horse) Serum (No. I 60) and SSS of Pneumococcus Type VI.

Amount of SSS (vol. 0.1 ml) mg	Anti-*H. influenzæ* serum (vol. 0.05 ml)			Anti-pneumococcus type VI serum (vol. 0.05 ml)			Saline solution (vol. 0.05 ml)		
	A	B	C	A	B	C	A	B	C
(1) 2	—	—	—	—	—	—	—	—	—
(2) 0.2	—	—	—	—	—	++	—	—	—
(3) 0.02	++	++++	++++	+++	++++	++++	—	—	—
(4) 0.002	+	++	++	+++	++++	++++	—	—	—
(5) 0.0002	—	—	—	++	++	++	—	—	—
(6) 0.00002	—	—	—	—	—	—	—	—	—
(7) 0	—	—	—	—	—	—	—	—	—

A = After 2 min.
B = After 10 min.
C = After 2 hr.
— = No precipitation.
+ to ++++ = Various degrees of precipitation.

with the original, non-absorbed serum. The results are summarized in Table II.

Table II reveals that absorption of anti-*H. influenzæ* horse serum with SSS of pneumococcus type 6 completely removed the precipitins against pneumococcus type 6, but did not noticeably affect the antibodies directed against *H. influenzæ* type b. Identical results were obtained in repeated experiments employing various amounts of SSS for absorption. Absorption of 0.3 ml of this anti-*H. influenzæ* horse serum with 0.2 mg, 0.02 mg, and 0.002 mg of SSS of pneumococcus type 6 resulted in complete elimination of the pneumococcal antibodies and did not decrease the reactivity of the serum with the polysaccharide of the influenza bacillus. It may be added that absorbed serum also failed to cause precipitation with the supernatant fluid of a pneumococcus type 6 brain heart infusion broth culture as antigen. It may be concluded, therefore, the anti-*H. influenzæ* horse serum contains two distinct antibodies, the one reacting with *H. influenzæ*, the other with pneumococcus type 6.

The question, then, arises as to whether or not the specific polysaccharide of *H. influenzæ* type b is antigenically related to the SSS of pneumococcus type 6. In order to elucidate this problem further, anti-*H. influenzæ* horse serum was absorbed with the specific polysaccharide of the type b influenza bacillus. To 0.5 ml of the serum was added 0.1 ml (0.098 mg) of the specific polysaccharide of *H. influenzæ*. Marked precipitation occurred. After incubation for 30 minutes at 37°C the mixture was centrifuged and the perfectly clear supernatant fluid was then tested for the presence of antibodies against pneumococcus type 6 and *H. influenzæ*. The experiment revealed that absorption with the specific polysaccharide of *H. influenzæ* type b resulted in complete elimination of the pneumococcal antibodies and the precipitins reacting with the specific polysaccharide of the influenza bacillus. The absorbed serum failed to cause precipitation with SSS of pneumococcus type 6 in amounts ranging from 0.2 mg to 0.000002 mg. In contrast, the non-absorbed serum reacted with the specific soluble substance of the pneumococcus in amounts of 0.02 mg to 0.0002 mg. It may be added that the absorbed serum failed to cause precipitation with the supernatant fluid of a pneumococcus type 6 brain heart infusion broth culture as antigen.

The results of these absorption experiments seem to allow of the conclusion that the available specific polysaccharide of *H. influenzæ* type b shares some antigenic component or part thereof with the available specific soluble substance of pneumococcus type 6. Furthermore, these findings throw some light on the possible origin of the type 6 pneumococcal antibodies in anti-*H. influenzæ* horse sera. The fact that the type b polysaccharide of the influenza bacillus absorbed the antibodies to the SSS of pneumococcus type 6 renders it likely that these antibodies were engendered

TABLE II.

Reaction Between Anti-*H. influenzæ* (Horse) Serum, Absorbed with SSS of Pneumococcus Type VI, and Specific Polysaccharide of *H. influenzæ* Type B.

Specific polysaccharide of *H. influenzæ* (vol. 0.1 ml) mg		Absorbed serum (vol. 0.05 ml)			Non-absorbed serum (vol. 0.05 ml)		
		A	B	C	A	B	C
(1)	0.098	++++	++++	++++	++++	++++	++++
(2)	0.0098	++	+++	+++	++	+++	+++
(3)	0.00098	—	—	—	—	—	—
(4)	0	—	—	—	—	—	—
SSS of pneumococcus type VI (vol. 0.1 ml) mg		A	B	C	A	B	C
(1)	0.2	—	—	—	—	+	+
(2)	0.02	—	—	—	+	+++	+++
(3)	0.002	—	—	—	—	++	++
(4)	0.0002	—	—	—	—	—	—
(5)	0	—	—	—	—	—	—

A = After 2 min.
B = After 10 min.
C = After 30 min.
— = No precipitation.
+ to ++++ = Various degrees of precipitation.

by *H. influenzæ* and that they are not due to the unintentional immunization with pneumococcus. The demonstration of an antigenic relationship between the specific substances of pneumococcus type 6 and the influenza bacillus type b serves as another example of common antigenic components in otherwise unrelated microörganisms.

Summary. (1) Certain anti-*H. influenzæ* horse serum reacts with SSS of pneumococcus type 6. Quantitatively, the precipitation reaction parallels that obtained with anti-pneumococcus type 6 serum. (2) SSS of pneumococcus type 6 absorbs the homologous antibodies, but does not affect the antibodies against the specific polysaccharide of *H. influenzæ* type b. (3) Absorption of the serum with the polysaccharide of the influenza bacillus removes both homologous antibodies and precipitins against the SSS of pneumococcus type 6. (4) It is concluded that such an anti-*H. influenzæ* horse serum contains two distinct antibodies and that some antigenic relationship exists between the available specific polysaccharide of *H. influenzæ* type b and the available SSS of pneumococcus type 6.

The author wishes to express his sincere appreciation to Dr. Hattie E. Alexander, The Babies Hospital, New York, for the supply of specific polysaccharide of *H. influenzæ;* to Dr. Edwin F. Voight, Lederle Laboratories, for SSS of pneumococcus type 6; to Dr. Leah Seidman, Harvard Medical School, Boston, Massachusetts, and to Dr. Morris Shaffer, Massachusetts State Antitoxin Laboratory, for anti-*H. influenzæ* horse sera.

14126

Rapid Method for Obtaining Anti-Rh Serum from Guinea Pigs.

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In order to prepare anti-Rh serum, Landsteiner and Wiener[1] recommended that guinea pigs be inoculated intraperitoneally with a suspension of washed *Rhesus* monkey erythrocytes equal to 2 cc of whole blood. The animals received 2 such injections 5 days apart, and were bled from the heart 7 days after the second inoculation. They stated that although most of the sera obtained in this manner could be used to show differences between Rh (+) and Rh (—) qualities of human blood, only "one or more" sera of a group of 10 animals thus immunized could be used diagnostically to determine the Rh characteristic. Recently these workers[2] suggested that the immunization of guinea pigs be accomplished by 5 successive inoculations of a suspension of *Rhesus* erythrocytes equal to 2 cc of whole blood given at 5-day intervals, and the sera collected by bleeding 7 days after the last inoculation.

In the present investigation anti-Rh sera were prepared according to 3 technics of immunization. The first anti-Rh serum was prepared according to the method suggested by Landsteiner and Wiener,[1] using adult guinea pigs weighing between 300 and 500 g. Twenty animals were given 2 intraperitoneal inoculations 5 days apart and were bled from the heart on the seventh day following the second inoculation. Two animals died during the immunization period from accidental causes, the remaining 18 yielded 3 diagnostically usable anti-Rh sera. Five of the 18 guinea pigs in this group were not exsanguinated during the bleedings, but were saved for further immunization. Six weeks after the first series of inoculations, these animals were given 2 doses of 2 cc of *Rhesus* blood at 5-day intervals and were bled 7 days following the last injection. Two sera of high titer and one serum of fair titer were obtained from these animals.

The second anti-Rh serum was prepared according to a modified method suggested by Landsteiner and Wiener.[2] A group of 16 adult guinea pigs weighing from 300 to 500 g were inoculated intraperitoneally with a suspension of erythrocytes equal to 2 cc of *Rhesus* blood. The animals were to be subjected to 5 separate inoculations 5 days apart. They were to be bled on the seventh day following the last inoculation. Nine animals failed to survive, 2 died within 4 hours, 4 died within 24 hours, and 3 died within 48 hours following the third inoculation. Post-mortem examinations revealed definite edema of the lung in all of these animals and it was concluded that death was due to hypersensitization. The administration of *Rhesus* monkey blood was discontinued for the 7 surviving animals. These were bled on the seventh day following the third inoculation. Three sera of high titer were obtained.

Although the modified technic of immunization (Landsteiner and Wiener) produced a high percentage of diagnostic sera in the animals surviving the third inoculation, the number of deaths from hypersensitization renders this method expensive.

The third anti-Rh serum was prepared by altering the above technics as follows: adult guinea pigs weighing from 300 to 500 g were given 5 intraperitoneal inoculations of *Rhesus* monkey erythrocytes in increasing doses at 48-hour intervals. The initial inoculation consisted of washed *Rhesus* monkey erythrocytes equal to 1 cc of whole blood. The inoculum was increased by 0.5 cc with each succeeding inoculation until the fifth or final inoculation, which consisted of 3.0 cc of washed erythro-

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* Assisted by a grant from the Christine Breon Fund.

[1] Landsteiner, Karl, and Wiener, Alexander S., *J. Exp. Med.*, 1941, **74**, 309.

[2] Wiener, A. S., personal communication.

cytes. The animals were bled from the heart on the second day following the last inoculation. Five of 9 animals used in this group produced reliable diagnostic sera, and there were no deaths among them.

All of the anti-Rh guinea pig sera were prepared as suggested by Landsteiner and Wiener[1], and stored in the refrigerator. The serum from each immunized guinea pig was diluted 0.1 cc of serum in 0.9 cc of 0.85% buffered saline. Immediately before use each was absorbed for one hour at room temperature with 0.05 cc of washed human erythrocytes of group A and 0.05 cc of washed human erythrocytes of group B. After absorption and before using the serum as a test reagent, it was further diluted one to 3 with 0.85% buffered saline. Diagnostic tests were made using known Rh (+) and Rh (—) human erythrocytes as controls. In each test 2 drops of the diluted guinea pig serum were placed in a 7 mm test tube to which was added one drop of a 1.5% suspension of washed human erythrocytes. The mixture was allowed to remain at room temperature for one hour before the sediments were read. Sera giving clear-cut Rh negative and Rh positive sediments were considered reliable for diagnostic tests.

Summary. A modified rapid method for the preparation of diagnostically suitable anti-Rh serum obtained from guinea pigs is described.

14127

Measurement of Circulation Time (Photoelectric Method).

BENJAMIN JABLONS AND JULES COHEN. (Introduced by S. Ansbacher.)

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Various methods have been proposed for the objective determination of the circulation time. This time is usually considered to be that which elapses between the injection of a particle of a foreign substance into the circulatory system and its arrival at the point of detection.

Many previous studies of the circulation time have been made with dyes. Meyer[1] first used blood containing methemoglobin as an indicator. Methylene blue was employed in animals by Stewart.[2] Koch,[3] Lian and Barras,[4] as well as Wollheim and Lange[5] employed fluorescein in humans. Thompson *et al.*[6] and Moore and Kinsman[7] used brilliant vital red for the study of the circulation time. Hamilton *et al.*[8] made use of phenol-tetra-iodophthalein sodium and Klein and Heinemann[9] employed congo red. Fishback[10] recently reported his experience with rabbits injected with fluorescein and other fluorescent substances.

Recently, we adapted a photoelectric method to the measurement of circulation time, the basic phenomena of which have been reported.[11] The principle involved is that a photoelectric cell is influenced by light transmitted through animal tissues, the intensity of which may be diminished by intravenously injected dyes.

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[1] Meyer, L., *C. R. Soc. Biol.*, 1892, **9**, 463.

[2] Stewart, G. N., *Am. J. Physiol.*, 1921-22, **58**, 27.

[3] Koch, E., *Deut. Arch. klin. Med.*, 1922, **140**, 39.

[4] Lian, M. C., and Barras, E., *Bull. Soc. Med. Hop. Paris*, 1939, **54**, 157.

[5] Wollheim, E., and Lange, K., *Verhandl. deut. Ges. inn. Med.*, 1931, **43**, 133.

[6] Thompson, W. O., Alper, J. M., and Thompson, P. K., *J. Clin. Invest.*, 1928, **5**, 605.

[7] Moore, J. W., and Kinsman, J. M., *J. Lab. and Clin. Med.*, 1939, **22**, 165.

[8] Hamilton, W. V., Moore, J. W., Kinsman, J. M., and Sperling, R. G., *Am. J. Physiol.*, 1928, **84**, 338.

[9] Klein, O., and Heinemann, J., *Zentrbl. inn. Med.*, 1929, **50**, 490.

[10] Fishback, D. B., *J. Lab. and Clin. Med.*, 1941, **26**, 12.

[11] Jablons, B., *Science*, 1943, in press.

Certain non-toxic dyes, such as 2 to 5 ml of a 1% solution of methylene blue or 5 ml of 0.5% Evans' blue, when injected intravenously, act as screens impeding the transmission of light, as observed by the deflection of the indicator of a sensitive galvanometer connected with a photoelectric cell. The time elapsing between the injection of the dye into the vein of the arm or leg, and its arrival at the point where the light and photoelectric cell have been placed, is determined by a stop watch, or can be recorded by connecting the leads from the photoelectric cell to a recording galvanometer.

In our studies, the circulation time was measured in humans from arm vein to ear lobe, and in rabbits from the ear vein of one ear to the opposite ear, using methylene blue almost exclusively. In humans, all determinations were made in the supine position. The ear unit was placed on either ear with the light source behind the ear. As the light was turned on, the needle of the galvanometer indicated the amount of current generated by the light transmitted through the tissues. Methylene blue was then injected rapidly into the arm vein, which was about at the level of the heart. The arrival of the dyed blood at the point of detection was thus indicated by a definite deflection of the needle of the galvanometer.

Objective determinations of the circulation time are made possible by this procedure, without the disadvantages inherent in other methods. Moreover, we have observed no venous thrombosis, as has been reported in other procedures. A few precautions, however, need emphasis. The needle employed should be of a sufficiently large bore (No. 18-20 gauge) to permit rapid injection of the dye. Methylene blue (1% solution) rarely produces a reaction even when injected in less than 1 to 2 seconds. In some individuals, 2 ml is sufficient to produce a deflection of the needle; however, in large subjects and patients with congestive heart failure, it has been found advisable to use as much as 5 ml. After the needle is in the vein, it is necessary to wait a few seconds before injecting the dye to stabilize the indicator on the galvanometer.

This method was found effective in animals and in humans. In normal rabbits the circulation time was approximately 6 seconds. In a series of 50 humans we obtained the following results:

Group A. In 14 patients without evidence of cardiac involvement the time varied from 9 to 16 seconds.

Group B. In 18 patients suffering from cardio-vascular disease (rheumatic heart disease, hypertensive cardio-vascular disease and coronary heart disease) who showed no subjective or objective evidence of decompensation, the time was similar to that of group A, namely, 9 to 15 seconds.

Group C. In 15 patients with decompensated cardio-vascular disease the time ranged from 20 to 45 seconds.

Group D. In 3 patients with hyperthyroidism the time ranged from 8 to 9 seconds.

All patients were in the 2nd to 8th decades of life, the majority in the 4th, 5th and 6th.

Summary. An objective method for the determination of circulation time is presented, which appears to have many advantages over previous methods with respect to accuracy, safety and facility to obtain recorded data. The use of an intravenously injected dye and a light source transmitted through animal tissue to a photoelectric cell, makes determinations possible, by noting the deflection of the needle in a sensitive galvanometer or other sensitive measuring device. The time elapsing between the injection of the dye and the deflection of the needle is the circulation time. Under experimental conditions, the normal circulation time ranged from 9 to 16 seconds, and in decompensated cardio-vascular disease as high as 45 seconds was recorded.

14128

Relation of Milk, Cholesterol and Estrogens to Cholic Acid Formation in Chicks.

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Liquid milk products in the diet of the chick are known to exert a preventive effect on the development of gizzard erosions caused by either a dietary deficiency or the presence of cincophen in the diet.[1,2] This preventive action of milk products is evidently related in both cases to an enhanced secretion of cholic acid.[2] An earlier report linked the gizzard erosions with a deficient formation of bile in the chick and especially of cholic acid in the bile.[3]

Further studies have been made of the action of milk in promoting cholic acid formation in the chick. In general, the methods employed were the same as previously described.[2,3] Two types of diets were used, a complete practical diet containing 1% of cincophen and a deficient basal diet.[2,3]

Attempts were also made to concentrate the anti-erosion and cholic acid-producing activity from whey by a procedure essentially similar to those used for the extraction of estrogenic substances from urine.[4] The extract obtained yielded distinct color tests for estrogens.[4,5] Presence of such substances in milk and whey has been reported.[6] We have, consequently, investigated known pure estrogenic compounds, since the presence of estrogens in milk and their general structural relation to the bile acids are particularly suggestive of a possible role in bile acid formation in the chick.

The results given in Table I confirm an

TABLE I.
Effect of Milk Products, Cholesterol, and Estrogens on Cholic Acid Content of Chick Gallbladder Bile.

Diet	Supplement	Level in diet	No. of groups	Avg cholic acid per g dried bile, mg
Practical	None		1	148
”	Skim milk	*ad lib.*	1	240
”	Cincophen	1%	1	118
”	Skim milk + cincophen	*ad lib.* 1%	1	203
Basal	None		6	104
”	Skim milk	*ad lib.*	4	168
”	Protein-free milk	”	3	154
”	Buttermilk	”	1	153
”	Lactic acid whey	”	1	155
”	Cholesterol	0.3%	2	100
”	Whey estrogen fraction	twice *ad. lib.* intake of whey	1	91
”	Theelin	100 mg/kilo	2	106
”	”	50 ”	1	98
”	Estriol	50 ”	1	105
”	Diethylstilbestrol	200 ”	3	80
”	”	50 ”	1	108

[1] Cheney, G., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 190.

[2] Almquist, H. J., Mecchi, E., and Kratzer, F. H., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 525.

[3] Almquist, H. J., and Mecchi, E., *J. Biol. Chem.*, 1938, **126**, 407.

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[5] Kleiner, I. S., *J. Biol. Chem.*, 1941, **138**, 783.

[6] Supplee, G. C., *Ind. Eng. Chem.*, 1940, **32**, 238.

earlier report[2] that milk products given in place of drinking water lead to an increased level of cholic acid in the gallbladder bile of the chick. In addition, it is shown that liquid buttermilk, whey and protein-free milk all exhibit an activity similar to that of skim-milk. The protein-free milk was made by isoelectric precipitation of casein, filtering, coagulation of remaining proteins by heating, filtering again and neutralizing. There remains no doubt that the greater part of the cholic acid-producing activity remains in milk after separation of the proteins of milk, and that the activity is resistant to heating to the boiling point at pH 4 to 5. Cholic acid-producing activity is not shown by the estrogen fraction from whey, or by the pure estrogens tested, or by another structurally related substance, cholesterol.

The results obtained in this study confirm previous reports of the existence in milk of a cholic acid-producing substance, possibly a precursor of bile acids, which is yet unidentified.

The authors are indebted to Dr. Oliver Kamm, Parke, Davis and Co., for a gift of theelin used in this work, and to Dr. T. H. Jukes, Lederle Laboratories, for the diethylstilbestrol.

14129

Effects of Smallpox Vaccination (Vaccinia) on Serologic Tests for Syphilis.

Grant O. Favorite.

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False positive serologic tests for syphilis have been attributed to many causes. More recently,[1,2,3,4] smallpox vaccination (vaccinia) has been added to the list. Lynch[3] and his associates, in a controlled study, showed that false positive reactions occurred in 16% of 263 vaccinated subjects. The Kolmer, Kline, Hinton and Mazzini tests were employed. One or more of these tests were positive, varying in intensity from ± to ++++. The reactions appeared in 2 weeks and remained positive for 2 months in most instances, but a few persisted for 4 months and one for 5 months.

An opportunity for a similar study was presented to us recently.[5] A group of individuals (mostly medical students and nurses) was chosen, in whom a serologic test had been performed within a reasonable time previous to the vaccination. There were 202 individuals, ranging in age from 3 weeks to 84 years. Over one-half were between 20 and 30 years of age. Forty-four individuals developed a primary cutaneous (non-immune) reaction at the site of inoculation, 134 developed a local accelerated (vaccinoid) form, and 24 were immune.

Out of 202 individuals on whom a single blood examination was performed, 19 were found to be positive, ranging in intensity from ± to ++++ by one or more of the Kolmer, Kahn and Mazzini tests. Not all persons were tested at the same time. They were examined from 10 days to 57 days following the vaccination, but 93.6% were done before the forty-third day. One month following vaccination 25 persons that gave a negative reaction were re-examined. Four out of this group were now positive. Undoubtedly, others would have been detected if the entire group had been examined more frequently.

In all, there were 24 individuals out of 202 who gave a false positive reaction for syphilis. This is an incidence of 11.8%. The majority of the reactions were ++ or less in intensity

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[2] Thomas, G. E., and Garrity, R. W., *U. S. Naval Med. Bull.*, 1941, **39**, 272.

[3] Lynch, F. W., Boynton, R. E., and Kimbal, A. C., *J. A. M. A.*, 1941, **117**, 591.

[4] Mohr, C. F., Moore, J. E., and Eagle, H., *Arch. Int. Med.*, 1941, **68**, 1161.

[5] Favorite, G. O., *Phila. Med.*, in process of publication.

TABLE I.
Distribution and Degree of Intensity of False Positive Reactions for Syphilis Following Smallpox Vaccination.

Time following vaccination	14 days			30-43 days			60 days		
Patient's No.	Kolmer	Kahn	Mazz.	Kolmer	Kahn	Mazz.	Kolmer	Kahn	Mazz.
9	+	—	++	—	—	—	—	—	—
15	+	++	++	—	+++	+++	—	—	—
23	+	—	—				—	—	—
25	—	—	—	±	—	—	—	—	—
32	—	—	—	—	++	++	—	—	—
33	—	—	—	—	+	—	—	—	—
35	—	—	—	—	—	++	—	—	+
52	—	—	++				—	—	—
55	++	—	+				—	—	—
58	—	+	++				—	—	—
78				—	±	+++	—	—	—
84				—	—	+++	—	—	+
102	++++	++++	+++	++++	++++	+++	—	++	+++
103				—	+	±	—	—	—
110				—	+	±	—	±	±
118				+	—	±	+	—	+
135				—	+	+	—	±	+
139				—	—	±			
146				—	—	++	—	—	++
147				—	—	+	—	—	—
150				±	—	—	—	—	—
178				—	—	+	—	—	—
203				—	±	—	—	—	—
204	++++	++++		—	+	±	—	±	—

TABLE II.
Summary of Number and Degree of Intensity of Reactions Encountered in 202 Individuals Following Smallpox Vaccination.

Degree of reaction	±	+	++	+++	++++	Total
Kolmer	2	5	1	—	3	11
Kahn	5	6	3	1	3	18
Mazzini	6	9	8	6	—	29
Total	13	20	12	7	6	58

but 10 were +++ or stronger in one or more tests. The reactions were about equally divided between the non-immune and the accelerated (vaccinoid) groups. None occurred in the immune.

Table I shows the distribution and the degree of intensity of the reactions encountered. Those individuals that were positive at the end of 14 days following vaccination were still positive (with one exception) at the end of 30-43 days. Fifteen became negative in 60 days, while 8 still gave positive reactions but to a lesser degree.

Table II shows the number and the degree of reactions of the tests employed. Seventy-eight percent gave a reaction of ++ or less, while 22% were +++ or stronger.

Comment. To show that the incidence of false positive reactions in this study was not due to chance alone, a survey was made of the serologic tests performed previously in the same laboratory. Including all tests, without regard to their specificity, the incidence of positive reactions was 0.9% in 1000 Kolmer and 1000 Kahn premarital examinations. For the control series for the Mazzini test, 1000 consecutive blood donors from the general population were used. There were 2.4% positive tests. Employing this figure for calculating the significant difference, when compared to the experimental group of 11.8% in 202 individuals, the difference is highly significant. Furthermore, the reactions persisted in the same individuals and gradually disappeared spontaneously. The vaccination was the only apparent factor operating.

Summary. Evidence is presented in confirmation of the work of others that smallpox (vaccinia) vaccination gives rise to false positive reactions for syphilis. The incidence of positive reactions by the use of the Kolmer, Kahn and Mazzini tests was 11.8%. The false positive reactions are transitory but may persist for 2 months or more.

Addendum. The patients with positive reactions were retested every two weeks. There was a gradual diminution in the intensity of the reactions until all became negative by the end of 120 days.

I wish to express my gratefulness to Miss Helen Breen and Miss Kathryn Simmons for their technical assistance.

14130

Growth of *Penicillium Notatum* on Various Media and the Development of an Antibacterial Substance.

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The antibacterial action of penicillium was first observed by Fleming,[1] and a further report was made by Chain and others.[2] Subsequently the preparation and the properties of this substance were reported in greater detail.[3,4]

The more recent studies have been primarily concerned with the isolation of a substance designated as penicillin and the mold was grown on a modified Czapek-Dox synthetic medium.[3] It seemed desirable to make some observations with this organism on other media. Various procedures were used to increase the growth of the penicillium and the effect of such procedures upon the production of an inhibitory substance was determined.

The data reported in this paper include (1) the growth of the mold penicillium on 3 different media (modified Czapek-Dox, infusion broth, and Amigen); and the relation of the pH of the media to the production of an antibacterial substance; (2) the influence of the addition of various sugars on the production of bacterial inhibitory substance; (3) the extraction of an antibacterial substance from the thallus.

Materials and Methods. Culture Media: (1) Modified Czapek-Dox synthetic media; this will be referred to as the Oxford Media. (2) Difco stock heart infusion broth; (3) an enzymic digest of purified casein and pork pancreas in which the proteins have been hydrolyzed to amino acids 75% and di- and tri-peptides 25%. This preparation will subsequently be referred to by its trade name of Amigen.* The pH range of all media was 4.4 to 5.0 when inoculated. All pH determinations were made with a glass electrode.

Cultures: A strain of *Penicillium notatum* was obtained from Dr. N. F. Conant, Durham, N.C. Stock cultures were grown on Sabouraud's media. The cultures for study were all grown in 225 cc Ehrlenmyer flasks. A strain of hemolytic *Staphylococcus aureus* isolated from a patient with osteomyelitis was used as the test organism. Dr. Gladys Hobby kindly determined the sensitivity of this culture to several preparations of penicillin. It was found to be about one-fourth as sensitive to the drug as the test organism, a strain of hemolytic streptococcus (C 203 MV).[5] This indicates that its sensitivity to penicillin is comparable to that of the strain of *Staphylococcus aureus* used by the Oxford workers.[6]

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[2] Chain, E., Florey, H. W., Gardner, A. D., Heatley, N. G., Jennings, M. A., Orr-Ewing, J., and Sanders, A. G., *Lancet*, 1940, **2**, 226.

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[4] Abraham, E. P., and Chain, E., *Brit. J. Exp. Path.*, 1942, **23**, 103.

* The media made with Amigen contained 10% of Amigen powder by weight dissolved in distilled water. The Amigen was supplied by Mead Johnson Co., Evansville, Indiana.

[5] Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 277.

Methods of Titration: (1) Serial dilution method. The material to be tested was diluted with broth in dilutions of 1:10, 1:20, 1:40 to 1:1,280. The tubes each containing 0.5 cc were then inoculated with the test organism using a 3 mm platinum loop (containing approximately 300,000 organisms) and incubated for 24 hours at 37°. The titrations seldom varied more than one tube. (2) Plate method: Melted blood agar was inoculated with hemolytic *Staphylococcus aureus* culture and poured into Petri dishes. Plugs of agar were removed with a 4 mm metallic cork borer, and 0.05 cc of the material to be tested was carefully placed in the openings. The plates were incubated for 24 hours and the diameter of the zone in which no growth occurred was measured. Observations made during this study indicate that the serial dilution method was the more satisfactory of the 2 methods.

Growth on Different Media. The mold penicillium grew well on both Amigen and broth when the initial pH was adjusted to 4.5. The growth was accelerated when grown on Amigen as compared with the other 2 media. The antibacterial titers did not persist for longer than 7 days in Amigen whereas they remained for a considerably longer period in broth. Growth was more uniform on broth and Amigen than on the Oxford media. Growth was slow at pH of 3 and there was none at a pH below 2.3. No change in pH occurred until growth became grossly apparent. The rate of growth then paralleled the increasing pH until the media became neutral; as the pH continued to rise, growth diminished.

Although no relationship was observed between the size of the thallus and the titer of the antibacterial substance, there was apparently some relationship between the character of the growth and titer. A slow-growing homogeneous thallus with a deep green, fine early sporulation that subsequently became green-brown in color was usually associated with a persistent titer in the media. Maximum titers were obtained usually between the fifth and eighth day when the pH of the media was between 7.0 and 8.0. The addition of hydrochloric acid to a growing culture was a marked stimulus to growth but temporarily delayed the development of an inhibitory titer of the culture media. The addition of acid to an alkaline culture without a titer reinstituted growth with subsequent rapid development of an inhibitory titer. When such an alkaline culture was acidified it rapidly became alkaline and there was renewed growth of the pellicle. This procedure was repeated several times and in each instance the titer which had fallen would again rise to its original level but it never exceeded the original titer. Subsequently, when the media was allowed to remain alkaline the titer rapidly disappeared.

The inhibitory titers of broth cultures persisted considerably longer than those of Amigen cultures. Titers of broth cultures have persisted for 20 days at a temperature of 2°C, whereas the titer of the same material disappeared in 4 days at room temperature. The titer of frozen broth media persisted for as long as 60 days.

Titers were enhanced by concentrating the broth culture media in a cellophane bag and hastening evaporation by an electric fan. There was a fair relationship between the titer and the degree of concentration (Table I).

TABLE I.
Effect of Concentration of Infusion Broth Cultures of Penicillium on the Inhibitory Titer.

Culture	Titer
Unconcentrated	1:160
Conc. to 50% original vol.	1:640
” ” 65% ” ”	1:1280
” ” 90% ” ”	1:20,480

The Influence of the Addition of Various Sugars to the Media. Experiments were set up to determine the effect of the addition of carbohydrate to the media. When sugar was added to broth media the pH remained acid for a longer period as growth progressed. The addition of sugar to broth or Amigen produced an increase in growth of penicillium in all instances.

Dextrose, sucrose, maltose and lactose were added to broth at pH 4.5, in concentrations of 1, 2, 5 and 10%. The media was then

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[6] Hobby, G. L., personal communication.

TABLE II.
Inhibitory Titers of Infusion Broth Cultures of Penicillium to Which Different Sugars Were Added.

	Days									
	4	6	8	10	12	14	18	22	26	30
Infusion Broth	80	320	80	80	80	10	—	—	—	—
” ” + 5% Lactose	40	160	320	640	640	1280	1280	640	320	160
” ” + 5% Maltose	0	0	80	160	640	80	80	—	—	—
” ” + 5% Sucrose	0	0	0	80	320	160	20	—	—	—
” ” + 5% Dextrose	0	20	80	40	20	—	—	—	—	—

inoculated and antibacterial titers and pH determined usually daily and occasionally every second day. The results will not be described in detail; however, of the various concentrations tested those with 5% sugar developed the highest inhibitory titers. A comparison of the different sugars is given in Table II. It is evident that of the sugars tried 5% lactose gave the maximum titer and that it persisted at a fairly high level for as long as 30 days. When lactose was added to Amigen or Czapek-Dox media it did not apparently enhance the inhibitory titers of penicillium cultures.

Bacteriostatic Substances Extracted from the Thallus. The thallus was removed from the culture media, dried carefully on filter paper and weighed. A small part was used for determination of dry weight and the remainder ground in a mortar with sea sand. The ground material was then immediately extracted with various solvents.

Extracts of the thallus in distilled water, ethyl ether, acetone or pyridine had no inhibitory effect upon the growth of *Staphylococcus aureus*. The ground thallus was extracted with small quantities of ethyl alcohol at 65°C. Much of the extracted material was inert and could be precipitated at 0°C. Equally active extracts were obtained at 25-40°C but they contained less inert material. When the thallus was dried to constant weight at 100°C prior to extraction, no bacteriostatic effect was noted in the alcoholic extracts.

The activity of such extracts was tested by the serial dilution method and it was found that 70 γ of crude alcohol soluble material was active against 300,000 organisms in 1 cc. Expressed in terms of Oxford units per mg the alcohol soluble material contained 0.095 Oxford units or approximately 0.1 of an Oxford unit per mg.

Alcohol extractions of the thallus were made successively from the 5th to the 11th day inclusive. The most potent extracts were obtained on thalli removed from 5- to 7-day-old cultures. The potency decreased on succeeding days and disappeared on the 10th day. No extracts of the thallus were active when the culture media on which the thallus grew failed to show some bacteriostatic property. At no time could a relationship be demonstrated between the amount of inhibitory substance in alcoholic extracts of the thallus and the titer of the culture media when the latter was active. There was no apparent relation between the quantity or rate of growth and the potency of the extracted material.

Conclusions. 1. *Penicillium notatum* grows well on nutrient broth and Amigen at pH 4.5. The growth is superior to that obtained on a modified Czapek-Dox medium. 2. The nature of the growth as well as the persistence of an antibacterial substance in the media is, in part, dependent upon the pH of the media. 3. Lactose added to broth cultures in a concentration of 5% considerably increased the inhibitory titer which persisted for as long as 30 days. 4. A bacteriostatic substance was extracted from the thallus of *Penicillium notatum* with ethyl alcohol.

14131

Study of Skin Histamine (With Some Results of Splanchnic Nerve Stimulation).*

Edward Lambert and Sol Roy Rosenthal.

From the Tice Laboratories, Research Department, Chicago Municipal Tuberculosis Sanatorium, and from the Departments of Pathology, Bacteriology and Public Health and Physiology, University of Illinois College of Medicine.

Rosenthal and Minard[1,2] devised a direct method of detecting diffusible histamine in the skin. The object of this paper is to present: 1. A modification of this method whereby extraneous substances which interfere with the assay of histamine can be avoided. 2. Preliminary experiments using this method to determine whether any alteration of the histamine content of the abdominal skin occurs following stimulation of the splanchnic nerve.

Method. Uniform pieces of skin about one centimeter in diameter and comparable in thickness to a Thiersch graft were placed between anode and cathode chambers for electrodialysis (Fig. 1). The raw surface of the skin was placed against a cellophane membrane cemented on the end of a glass tube, 8 mm inside diameter. The skin was held securely over the end of the tube by a rubber band. A rubber dam with a minute perforation was stretched so that the edge of the perforation reinforced the rubber band and protected the cut edges of the skin from the anode fluid. Three-tenths milliampere of current was passed through the skin for a period of 3 minutes. The cathode fluid, consisting originally of 0.2 ml of Ringer's solution, was removed and heated for 30 minutes at 100°C with 0.05 ml of normal hydrochloric acid. It was then evaporated to dryness to remove the excess hydrochloric acid and made up to 0.2 ml volume with distilled water. The pH was adjusted to that of Ringer-Locke solution with neutral red indicator. The samples thus obtained were assayed on a strip of guinea pig ileum in a 3.5 ml bath of atropinized (0.5 γ per cc) Ringer-Locke solution, using histamine hydrochloride as a standard.

Results. Samples of normal skin from the abdomen and chest of dogs, when treated as described, yielded an equivalent of 0.000 to 0.005 γ of histamine. The amount in each of several pieces of skin from the same animal was relatively constant.

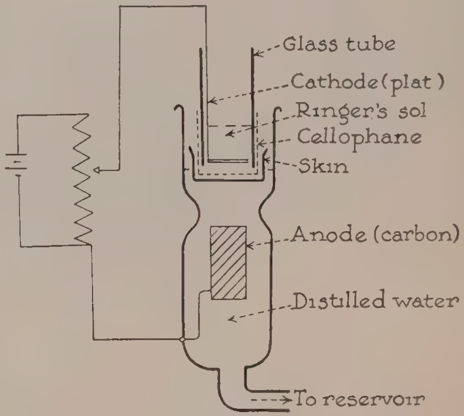

Fig. 1.
Apparatus for Electrodialysis of Skin Samples.

Small amounts of histamine (1:5,000,000 solution) injected into the skin increased the histamine equivalent of the cathode fluid. Burning of the skin resulted in a large increase of the histamine equivalent. Experiments in which turpentine, xylol or chloroform were applied to 15 samples of skin (intact or removed from body) for from 5 to 10-minute periods, indicated that these chemical irritants may also increase the histamine equivalent of the cathode fluid.

That the active substance in the cathode fluid is most probably histamine is indicated by the following facts: 1. It causes contraction

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* Aided partially by a grant from Abbott Laboratories.

[1] Rosenthal, S. R., and Minard, D., *J. Exp. Med.*, 1939, **70**, 415.

[2] Emmelin, N., Kahlson, G. S., and Lindström, K., *Acta Physiol. Scand.*, 1941, **2**, 78.

of the atropinized guinea pig ileum. 2. This activity is heat stable in acid. 3. It is heat labile in alkaline solution. 4. It is inhibited by thymoxyethyldiethylamine.

These observations demonstrate that, using the method described, very little histamine can be obtained from untreated dog skin. However, histamine injected into the skin or liberated in the skin by burning or chemical irritation can be readily detected.

2. The above technic for the study of skin histamine was devised originally to determine whether any alteration of the histamine content of the skin occurred in association with referred pain. Rosenthal and Minard[1] have presented evidence indicating that histamine is the chemical mediator of cutaneous pain. Pollack and Davis[3] and others have suggested that referred pain may be due to the action of metabolites released in the skin by viscerocutaneous reflex activity.

A preliminary study has been carried out with dogs to determine whether histamine is liberated in the skin of the abdomen following stimulation of the splanchnic nerve. Control samples of skin were taken from the right side of the upper abdomen and lower chest of dogs. Electrodes were then applied to the left splanchnic nerve under procaine or light ether anesthesia. Additional samples of skin were taken from the left side following the operation and after electrical stimulation of the left splanchnic nerve (Harvard inductorium, using 2 batteries; secondary coil at 10 cm or less). Both the control samples and those obtained following electrical stimulation were taken from scattered sites on the skin area mentioned above, since there was no evidence to indicate where maximal viscerocutaneous reflex activity might occur. Immediately after it was obtained, each sample of skin was subjected to the method of extraction described above and, later, was tested on the guinea pig ileum. The effect of electrical stimulation was tested from 4 to 10 times in each animal.

Fifteen experiments were carried out according to this procedure. Table I shows the results in an experiment which is typical of 3 of these. Fig. 2 is a portion of the assay from another of these. There was an increase in the histamine equivalent of the skin dialysate following the operation and, in each case, following stimulation of the splanchnic nerve. It is to be noted, however, that each sample of skin was taken in duplicate and that the dialysates of one set were not heated in acid as prescribed above. No change in the histamine equivalent was seen in the latter set. This suggests that there is in untreated dialysates either a diffusible substance which is activated by heating in acid, or a heat labile substance which inhibits the action of histamine on the guinea pig ileum. The fact that untreated dialysates of skin when added to the bath in which the gut is suspended frequently depress the activity of added standard histamine solutions favors the latter explanation.

TABLE I.
Histamine Equivalents (in γ) Obtained from the Skin following Splanchnic Stimulation.

Sample No.	Heated in acid	Not heated in acid	Controls
1, 2	.000	.000	
3, 4	.000	.000	
5, 6	.000	.001	
7, 8	.000	.000	
9, 10	.001	.000	Operation
11, 12	.000	.000	
12, 14	.0025	.001	Stimulation
15, 16	.002	.000	"
17	.0075		
18	.000		"
19, 20	.0035	.000	"
21, 22	.0035	.000	
23	.000		
24	.000		
25	.000		

Six of the remaining experiments were not as clear cut as that just described, but showed an increase in histamine equivalent in only occasional skin samples after splanchnic nerve stimulation. Six experiments showed no appreciable change in the histamine equivalent. Three control experiments were carried out in which there was no attempt to apply electrodes or stimulate the splanchnic nerve. No change in the histamine equivalent of the skin samples

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[3] Davis, Loyal, and Pollack, Lewis J., *Arch. Neur. and Psychiatr.*, 1932, **27**, 282.

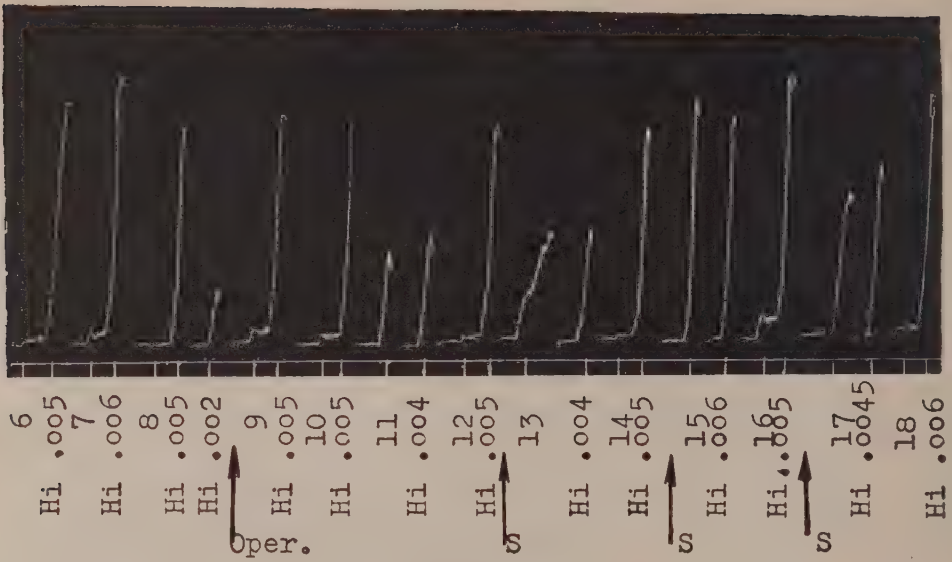

FIG. 2.
Effect on the ileum of standard histamine solutions (Hi., in γ) and of skin dialysates (samples 6-18). Samples 7 and 8, 9 and 10, etc., are duplicates. Odd numbered samples were heated in acid; even numbered samples, untreated. Oper, application of electrodes; S, stimulation.

occurred. However, a slight increase in the histamine equivalent of the skin samples occurs following application of the electrodes to the splanchnic nerve, without there having been an electrical stimulation. This may be due to irritation or mechanical stimulation of the nerve by the application of the electrodes.

Summary. A method for the study of skin histamine has been described. The histamine equivalent of skin extracts obtained by this method has been shown to increase following injection of histamine into the skin and following burning or chemically irritating the skin. That the substance studied is closely related to histamine has been shown by several tests. Using the method described, experiments have given suggestive evidence that histamine may be liberated in the abdominal skin upon the application of electrodes to and stimulation of the splanchnic nerve.

14132

The Neurenteric Canal in the Frog, *Rana Pipiens.*

ROBERTS RUGH

From Washington Square College of Arts and Science, New York City.

Even the temporary appearance of a tubular connection in the embryo between the neurocoel and the hindgut, known as the neurenteric canal, has considerable phylogenetic significance if its presence can be demonstrated and the parts homologized in such a variety of forms as Amphioxus, Fish, Amphibia, Reptiles, Mammals and possibly even in birds (duck, goose, Sterna., Lillie[1]). It is the purpose of this paper to add another link in the chain of evidence of the embryological existence of such a canal, using the Anuran, *Rana pipiens.*

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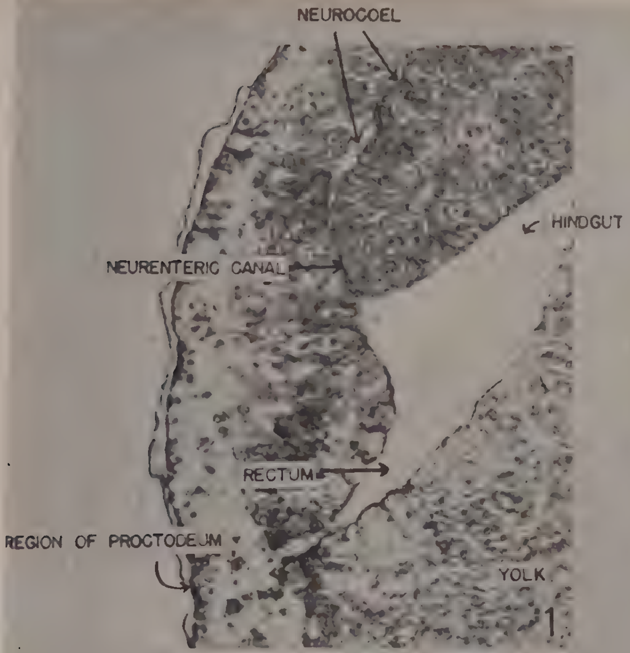
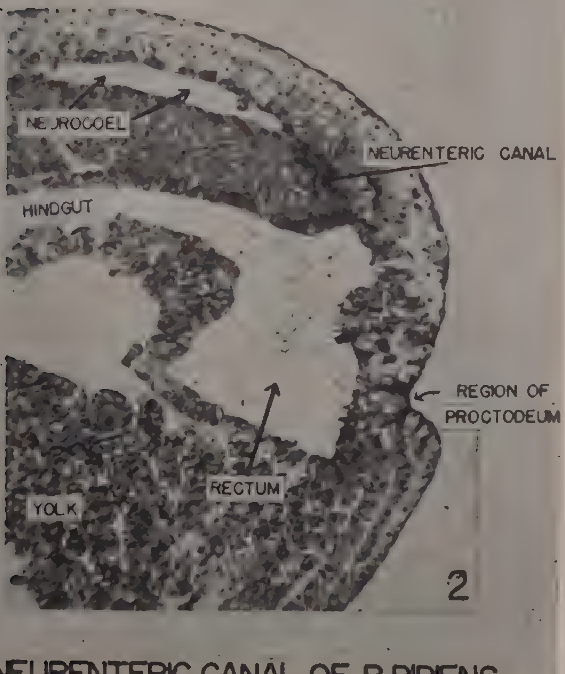
[1] Lillie, F. R., *The Development of the Chick,* Henry Holt Co., 1919.





NEURENTERIC CANAL OF R. PIPIENS

The neurenteric canal has been variously described as a tubular connection between the neurocoel and hindgut; the post-anal gut (Shumway[2] and Huettner[3]); the dorsal cavity of the closing blastopore; the short bent posterior portion of the neurocoel plus the remains of the blastopore; the notochordal canal; the anterior end of the primitive streak; and also the connection between the amniotic and yolk sacs of the mammal. Huxley and DeBeer,[4] for instance, are very cautious in stating that the blastopore becomes an enclosed tube with the approximation of the parallel neural ridges at that region so that "in this manner, a neurenteric canal, actual or virtual, according as to whether the blastopore is or is not still open, is formed, connecting the cavities of the neural tube and the gut."

Over 100 frog embryos (*Rana pipiens*) of stages 15 to 17 (Shumway[5] and Rugh[6]) were serially sectioned sagittally at 10 μ and examined for the possible existence of this canal. In practically every embryo the canal was either present as such (Fig. 1) or its lining of pigmented cells could be easily discerned (Fig. 2). Those embryos which had recently closed blastopores showed the most perfect neurenteric canals. In slightly older embryos (*i.e.*, stage 18) the neurenteric canal was occluded, but its earlier position could be determined by the column of pigmented cells, even though the neurocoel and hindgut were permanently separated.

An examination of the arrangement of cells in Fig. 1 will indicate the continuity of the neurocoel and the posterior archenteron and will also emphasize the differences, particularly in size, of the overlying posterior integumentary cells originally derived from the blastoporal lips.

There is no dispute as to the existence of this canal in Amphioxus where it persists through late larval life (Arey,[7] Conklin,[8] McEwen[9]). In Teleost Fish, Kupffer's vesicle is supposed to represent in part a rudiment of the neurenteric canal (McEwen[9]) even though a canal as such never develops. In Amphibia the available data refer only to the Anura and even here it is not consistent. Huettner[3] says, "This" (neurenteric) "canal does not exist in the embryo of the wood frog, at least not as a spacious passage between the neurocoel and the primitive gut. However, there are certain structures developed by the closure of the blastopore which may be considered as homologous to the neurenteric canal of Amphioxus." In the chick, McEwen[9] says, "Thus there is formed a typical neurenteric canal in just the region where such a canal would be expected to appear, were the primitive groove a blastopore," while Jordan and Kindred[10] state, "The neurenteric canal is derived by a perforation of the floor of the primitive pit." Lillie[1] says, "In the chick the neurenteric canal is never typically developed. Usually it is represented only by the primitive pit. In exceptional cases I have found traces of it in the head process." In the Mammal, McEwen[9] says, "It is perhaps doubtful whether the perforation, when present, even persists until the fusion of the neural folds in this region is actually complete. During its existence, however, the opening in question is so closely homologous with the neurenteric canal of the frog that it is frequently so named," and Jordan and Kindred state that "The primitive node" (of the mammalian embryo) "is pierced by a patent neurenteric canal, the homologue of the primitive pit."

It must be emphasized that this neurenteric canal as a tube is small; that it exists for a maximum of about 17 hours at 18°C; and that it can be seen only in stages No. 15 to

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[2] Shumway, W., *Introduction to Vertebrate Embryology*, John Wiley & Co., 1941.

[3] Huettner, A. F., *Comparative Embryology of the Vertebrates*, 1941.

[4] Huxley, J., and DeBeer, G. R., *The Elements of Experimental Embryology*, Macmillan & Co., 1934.

[5] Shumway, W., *Anat. Rec.*, 1940, **78**, 139.

[6] Rugh, R., *Experimental Embryology, a Manual of Techniques and Procedures*, N. Y. University Bookstore, 1941.

[7] Arey, L. B., *Developmental Anatomy*, W. B. Saunders Co., 1940.

[8] Conklin, E. G., *J. Morph.*, 1932, **54**, 69.

[9] McEwen, R. S., *A Textbook of Vertebrate Embryology*, Henry Holt Co., 1931.

[10] Jordan, H. E., and Kindred, J. E., *A Textbook of Embryology*, D. Appleton-Century Co., 1937.

No. 17 of early embryonic development. Its transient nature may be the reason for the denial, or questioning, of its existence in the various references mentioned. Nevertheless, it is the contention of this paper, based upon the study of many sectioned embryos during this particular period of development that

a. A neurenteric canal actually does exist in fact as a tubular connection between the neurocoel and the hindgut of the embryo of *Rana pipiens*.

b. The cells of the floor of the neurocoel, of the floor of the neurenteric canal, and of the roof of the archenteron are of the same type and are, in all probability, continuous in early development.

c. The post-anal gut and the neurenteric canal are not synonymous but may be continuous.

d. The neurenteric canal is very small, tubular, and transient. It is formed by the depression of the posterior medullary plate along with the dorsal lip of the blastopore, concurrently with coalescence of the posterior neural folds and the lateral lips of the same blastopore which together form the sides of the temporary neurenteric canal.

14133

Effect of Quinones on Acid Formation in Saliva.

W. D. Armstrong and J. W. Knutson.*

From the Laboratory of Dental Research, University of Minnesota, Minneapolis.

Fosdick, Fancher and Calandra[1] noted that a saliva-glucose mixture containing 1 mg per 100 cc of synthetic vitamin K (2-methyl-1,4-naphthoquinone) "forms no acid in a 4-hour incubation period, while the same mixture will produce up to 2 m.eq. of acid under the same conditions in the absence of the vitamin K." These authors emphasized that the effect on bacterial metabolism observed by them to be a property of synthetic vitamin K and discussed the possible uses of this compound in the prevention of dental caries.

The possibility suggested itself to us that the bacterial inhibitory action of synthetic vitamin K in saliva might be due to the fact that this compound is a quinone and that its antibacterial property has no actual relation to its vitamin K activity. It was already known[2] that benzoquinone possesses a substantial bacteriostatic ability. Recently reports[3,4] have appeared of a remarkable bacteriostatic effect exerted *in vitro* by certain dimethoxy quinones related structurally to fumigatin. Fumigatin (3-hydroxy,4-methoxy,2:5-toluquinone), produced by *Aspergillus fumigatus*, in itself possesses a definite ability to inhibit the multiplication of several gram-positive organisms.[5]

All points in Fig. 1 referring to a particular quinone were determined with solutions incubated concurrently using the same lot of pooled saliva to which 10% of its weight of glucose had been added (recommendation of Fosdick *et al.*[1]) Other experiments demonstrated that essentially the same results were obtained when only 2% of glucose was added to the saliva. Those quinones which were not sufficiently soluble in water were made up in alcohol, or chloroform in the case of 2,6-dimethoxy-benzoquinone, so that equal volumes contained equal molecular amounts of the several substances. The required volumes (0.05 to 1.0 cc) of these solutions were added to dry 25 cc volumetric flasks and the solvent evaporated under reduced pressure so that a

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* Passed Assistant Dental Surgeon, United States Public Health Service.

[1] Fosdick, L. S., Fancher, O. E., and Calandra, J. C., *Science*, 1942, **96**, 45.

[2] Graham-Smith, G. S., *J. Hyg.*, 1919, **18**, 1.

[3] Oxford, A. E., *Chemistry and Industry*, 1942, **61**, 189.

[4] Oxford, A. E., *J. Chem. Soc.*, 1942, p. 577.

[5] Oxford, A. E., and Raistrick, H., *Chemistry and Industry*, 1942, **61**, 128.

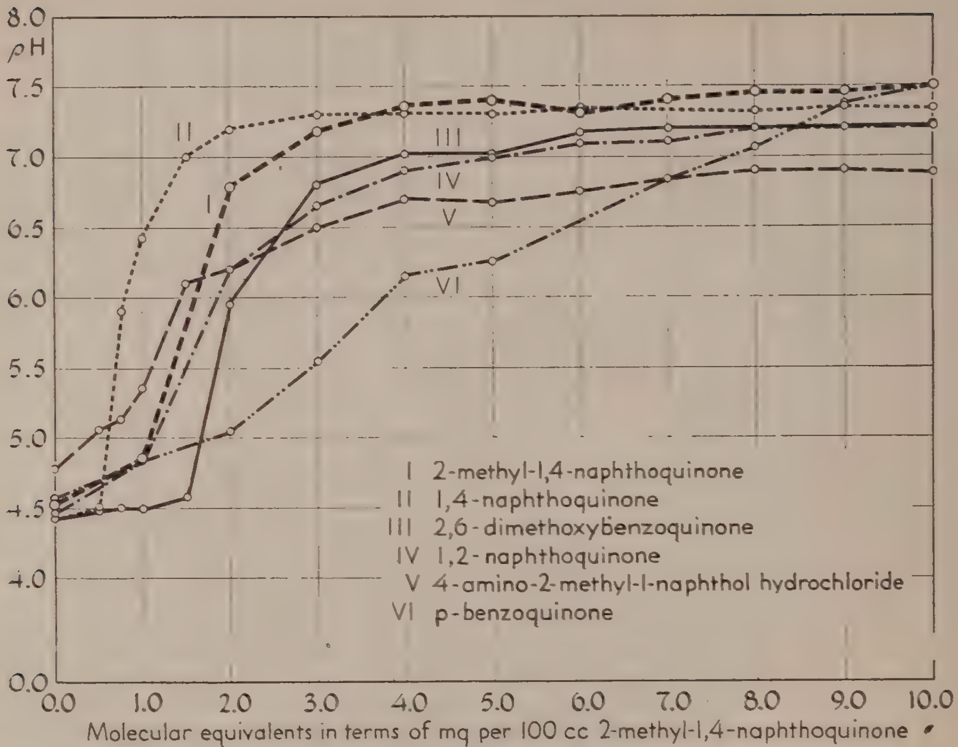

FIG. 1.

Inhibition of acid formation in saliva-glucose mixtures incubated with equal molecular concentrations of quinones.

film of the quinone was deposited over the wall of the flask. The substances soluble in water[†] were transferred in measured volumes (0.05 to 1.0 cc) to dry 25 cc volumetric flasks as aqueous solutions of the same molecular concentration as the alcoholic solutions referred to above. Sufficient water was added to all flasks to make the total volume of added water 1 cc. Immediately after adding 15 cc of the saliva-glucose mixture to each flask; and after the glass stoppers were tightly inserted, the flasks were incubated with constant shaking at 37° for 4 hours. Although some of the quinones are not directly soluble in water to a degree equivalent to the highest amounts added to the saliva, it was observed, in the case of clear broths and synthetic media, that the technic described above gave clear solutions of the quinones almost at once after the agitation was started. The flasks were allowed to cool to room temperature in a refrigerator and then shaken. The pH of the contents was determined, using a glass-electrode instrument, immediately after opening each flask. The quantities of 2-methyl-1,4-naphthoquinone used were in definite increments in terms of milligrams per 100 cc of the final dilution. It is to be noted throughout this paper that the other substances are compared with 2-methyl-1,4-naphthoquinone on a molecular basis. The data in Table I were obtained in the manner already described using the same lot of pooled saliva-glucose mixture.

[†] Benzoquinone, hydroquinone, 2-methyl-1,4-naphthoquinone sodium bisulfite addition compound (Hykione), 1-2-naphthoquinone-4-potassium sulfonate, sodium anthroquinone beta sulfonate, sodium-2-methyl-1,4-naphthoquinone disulfate and 2-methyl-4-amino-1-naphthol hydrochloride (Synkamin).

TABLE I.

Comparison of Inhibition of Acid Formation in Saliva-Glucose Mixtures by Substances in a Molecular Concentration Equal to 2 mg per 100 cc of 2-methyl-1,4-naphthoquinone.

Compound tested	pH
Unincubated saliva-glucose mixture	7.40 7.40
Untreated control	4.60 4.65
1,4-Naphthoquinone	7.35 7.35
2-Methyl-1,4-naphthoquinone	6.90 6.90
1,2-Naphthoquinone	6.85 6.80
2-Methyl-1,4-naphthoquinone sodium bisulfite addition compound*	6.45 6.45
Benzoquinone	5.60 5.45
Tolu-*p*-quinone	5.20 5.20
Quinhydrone	5.20 5.15
1,2-Naphthoquinone-4-potassium sulfonate	5.15 5.10
Hydroquinone	5.00 5.00
2-Hydroxy-1,4-Naphthoquinone	5.00 4.95
Tolu-*p*-hydroquinone	5.00 4.90
Sodium-anthroquinone-beta sulfonate	4.75 4.75
Sodium-2-methyl-1,4-naphthohydroquinone disulfate	4.65 4.70
Methylene blue	6.95 6.90
Sodium-2,6-dichlorobenzenone indophenol	5.00 5.00
Neutral red	5.30 5.15

* Abbott's "Hykinone."

The data in Fig. 1 indicate that while 1 mg of 2-methyl-1,4-naphthoquinone per 100 cc saliva effectively decreases acid formation to a degree below that required for decalcification of tooth structures, a concentration of upwards of 3 mg per 100 cc is required for complete inhibition of acid formation. Consideration of the data presented in this paper demonstrates that the inhibitory effect of synthetic vitamin K on acid formation in saliva, discovered by Fosdick *et al.*,[1] is merely incidental to its properties as a vitamin. It will be noted that 1,4-naphthoquinone, under the conditions of these experiments, has an antibacterial effect somewhat superior to that of 2-methyl-1,4-naphthoquinone; yet the first named substance is reported[6] to exhibit not more than 10% the vitamin K potency of the second. Furthermore, 1,2-naphthoquinone and benzoquinone whose vitamin K activities are negligible[6] are shown to possess in substantial degree an ability to interfere with acid formation from glucose by salivary organisms.

Oxford[3] has recently reported 2,6-dimethoxy benzoquinone to inhibit the growth of a certain strain of staphylococcus in dilutions of 1:400,000 and was inferior in this regard only to 4,6-dimethoxy tolu-quinone. It is to be noted (Fig. 1) that certain naphthoquinones equal or surpass, under the conditions of these experiments, the ability of 2-6-dimethoxy benzoquinone to interfere with the metabolism of salivary organisms. The results obtained (Fig. 1) with 2-methyl-4-amino-1-naphthol hydrochloride (Synkamin of Parke Davis) are to be explained by its ready oxidation to the corresponding 1,4-naphthoquinone, a fact which also accounts for its vitamin K activity.

It might be supposed that there could exist a relationship between the oxidation-reduction potentials of quinones and their relative effectiveness as bacteriostatic agents. While the information at hand will not allow this question to be settled, a consideration of the data in Table I in relation to the E_0 values of the several substances makes it appear probable that some character of quinones other than their oxidation-reduction potentials is also determinant of their antibacterial properties. The data referring to the 3 oxidation-reduction indicators appended as the last 3 items of Table I were obtained in an investigation of this point. The marked effect produced on antibacterial ability by substitution in the quinoid ring is seen when one compares the results obtained with 1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone and those obtained with 1,2-naphthoquinone and 1,2-naphthoquinone-4-potassium sulfonate.

Fosdick *et al.*[1] indicated that 2-methyl-1,4-naphthoquinone in low concentrations has no effect on bacterial growth. We found that 3 and 5 mg of this quinone per 100 saliva-glucose mixture and corresponding amounts of benzoquinone caused a progressive decrease in the *Lactobacillus acidophilus* counts in samples withdrawn at hourly intervals over a 4-hour incubation period. The final count was reduced to about one-third the original number of organisms while that of untreated controls increased by about 30%.

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[6] Thayer, S. A., Cheney, L. C., Binkley, S. B., MacCorquodale, D. W., and Doisy, E. A., *J. Am. Chem. Soc.*, 1939, **61**, 1932.

Several of the naphthoquinones mentioned above have been found to inhibit in high dilution the growth of staphylococcus, streptococcus, and pneumococcus in Gladstone's medium and in broth. Quantitative data as to the bacteriostatic and lethal concentrations required against these organisms in several media are now being obtained.

This work was aided by a grant from the William John Gies Fund of the American College of Dentists. We also wish to express our thanks for gifts of material used in this work as follows: Dr. Louis F. Fieser, Harvard University (2-hydroxy-1,4-naphthoquinone and 1,2-naphthoquinone-4-potassium sulfonate); Dr. Richard Kohn-Richards, Abbott Laboratories (Hykinone and 2-methyl-1,4-naphthoquinone); Dr. Oliver Kamm, Parke Davis (Synkamin); Dr. Walter M. Lauer, University of Minnesota (2,6-dimethoxy benzoquinone); and Dr. Karl Folkers, Merck and Company (sodium 2-methyl-1,4-naphthohydroquinone disulfate).

14134 P

Growth of *Brucella* in a Simple Chemically Defined Medium.

N. B. McCULLOUGH AND LEO A. DICK. (Introduced by Gail M. Dack.)

From the Brucellosis Research Laboratory, The Clayton Foundation, University of Texas, Austin.

Zobell and Meyer[1] studied the metabolism of the *Brucella* in simplified media. In the absence of knowledge of the accessory growth factors required by this group only slight growth was obtained when such media were used. They were able however to show that certain strains of *Brucella* could utilize the ammonium ion as a source of nitrogen and various organic acids as a source of carbon and energy. They did not find that the simple sugars, including glucose, could serve as the sole source of carbon and energy. McNutt and Purwin[2] found that slight growth of some strains occurred with arabinose or xylose as the sole source of carbon. It is worthy of note that in these instances ingredients were used which are now known to carry various accessory growth factor substances as contaminants.

Koser, Breslove and Dorfman[3] were the first workers to obtain luxuriant growth of the *Brucella* in a chemically defined medium by the addition of known accessory growth factor substances. Their medium consisted of 17 amino acids, glucose, inorganic salts, and the appropriate accessory factors. Following the elucidation of at least some of the accessory growth factor requirements of this group it has become possible to obtain abundant growth of some strains of *Brucella* in a very simple chemically defined medium.

Eight strains of *Brucella,* 3 each of *Br. abortus* and *Br. suis,* and 2 of *Br. melitensis,* were used in this preliminary work. Five of these were stock laboratory strains obtained originally from Dr. I. F. Huddleson and from the State Department of Health, Austin, Texas. Two of the strains of *Br. abortus* were recent isolations from Texas dairy cattle, and one strain of *Br. suis* was an isolation from a human case of undulant fever one week prior to its use in this study. All strains grew well in a normal atmosphere.

The inorganic salts used were of C.P. grade. The glucose was the grade usually used in bacterial fermentation tests. The accessory factors were pure crystalline preparations. The biotin was a solution of crystalline biotin (free acid) obtained from the S.M.A. corporation. The various ingredients were dissolved in redistilled water and sterilized by filtration through a Seitz filter. Growth tests were performed as described in a previous paper.[4] For initial seedings the inocula ranged between 4,000 and 25,000 cells per cc as judged by plate counts.

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[1] Zobell, C. E., and Meyer, K. F., *Science,* 1930, **72**, 176; *J. Infect. Dis.,* 1932, **51**, 344.

[2] McNutt, S. H., and Purwin, P., *J. Infect. Dis.,* 1931, **48**, 292.

[3] Koser, S. A., Breslove, B. B., and Dorfman, A., *J. Infect. Dis.,* 1941, **69**, 114.

After preliminary experiments to determine the optimum amounts of the various ingredients the following medium was devised:

Ammonium Sulphate	0.5	g
Sodium Chloride	7.5	’’
Dipotassium Phosphate	1.0	’’
Magnesium Sulphate	0.1	’’
Sodium Thiosulphate	0.1	’’
Glucose	1.0	’’
Thiamine Chloride	200	μg
Nicotinic Acid	200	’’
Calcium Pantothenate	40	’’
Biotin	1.0	’’

Redistilled water to make 1,000 cc.
pH adjusted to 6.8 to 7.2.

Of the 8 strains used 7 grew well in this medium; although growth was slower the maximum amount of growth obtained was comparable to that obtained in Tryptose broth. With the stated seeding growth usually appeared within 42 to 60 hrs and became maximal within 6 to 7 days. One strain of *Br. melitensis* failed to grow in this medium even when large seedings were used. It is of interest that this strain likewise failed to grow in Koser's amino acid medium. The 7 strains that grew successfully were carried through 10 serial transplants. Excellent growth was maintained throughout the series. They were then examined with the usual morphological, biochemical and immunological tests for conformity to the characteristics of the respective species. All presented typical smooth colonies and all could be readily typed by means of the tests used. For the immunological tests absorbed typing sera were used. These sera reacted to full titer with the appropriate strains.

Other sources of ammonium ion are satisfactory; however when potassium nitrate was used as sole source of nitrogen no growth of any strain occurred. This may possibly be due to the effect of this compound on the Eh of the medium. In the absence of sodium thiosulphate only slight irregular growth of a few strains occurred. Many other sources of carbon and energy may be used to replace glucose. A subsequent paper will deal with this question. The medium may be sterilized in the autoclave at 15 lbs for 15 min without impairing its growth promoting properties.

This medium is inexpensive and lends itself readily for the further investigation of accessory growth factors required by strains of this group as well as to other physiological problems concerning the *Brucella*. Utilization of carbon compounds can be judged by success or failure to obtain growth rather than by less critical methods which must necessarily be employed when complex media are used.

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[4] McCullough, N. B., and Dick, L. A., *J. Infect. Dis.*, 1942, **71**, 193.

14135

Failure to Induce Ovulation in Constant Estrous Guinea Pigs.*

F. L. Dey. (Introduced by W. F. Windle.)

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Ovulation may be induced in the rabbit, which normally ovulates only following copulation, by injecting a dilute solution of copper acetate either intravenously[1] or else into the third ventricle.[2] This reaction fails to occur after transection of the hypophysial stalk.[3] Acetylcholine, on the other hand, induces ovulation in the rat regardless of whether the stalk is intact or not.[4]

Guinea pigs with large lesions in the anterior hypothalamus at the caudal border of the optic chiasma fail to ovulate post-operatively.[5] The vaginal membranes of these animals remain constantly open and the genitalia increase markedly in size. The ovaries resemble those of the pre-copulatory rabbit, *i.e.*, ovarian follicles develop but ovulation does not occur spontaneously and corpora lutea are not formed. This disturbance of genital function appears to be due to the failure of the pituitary to release luteinizing hormone. When this hormone is administered artificially, the ovarian follicles rupture, corpora lutea are formed and the vaginal membrane closes. A single injection of luteinizing hormone is sufficient to cause this reaction. The vaginal membrane remains closed for an entire cycle (13-16 days) once luteinization is effected.[6]

Experiments were undertaken to determine whether luteinization might be induced in guinea pigs with this type of disturbance by injecting acetylcholine or copper acetate.

Eight mature female guinea pigs were utilized in this study. Large electrolytic lesions were placed in the anterior hypothalamus of each animal by means of the Horsley-Clarke stereotaxic instrument. Following this procedure, the animals developed the condition of constant estrus. The injection experiments were begun some 3 weeks after the onset of this condition. In one animal, 7.5 mg of copper acetate in normal saline (10 mg/cc) was injected into the jugular vein; in 2 animals, 0.05 mg of copper acetate (in 0.01 cc of saline) was injected into the third ventricle; and in a fourth animal, 0.05 mg of the drug (in 0.01 cc of saline) was injected directly into the pituitary gland. In the 4 remaining animals, a mixture of acetylcholine and physostigmine (10 γ acetylcholine plus 5 γ physostigmine in 0.002 cc saline) was injected directly into the pituitary gland. Following these injections, the animals were observed daily for a period of 9 to 12 days and the condition of the vaginal membrane was recorded. The animals were sacrificed at the end of this period and the ovaries were prepared for microscopic study.

The vaginal membrane of only one of the 4 animals injected with copper acetate closed post-operatively. In this animal, the drug had been injected directly into the hypophysis. The animal became quite ill following the injection and it was killed 7 days later. The vaginal membranes of each of the 4 animals injected with acetylcholine were reduced to a slight tear 4 days after the injections, but they opened widely again within 3 days. The ovaries of all the animals utilized in the experiment were studied microscopically and no evidence of ovulation or corpus luteum formation was found.

These experiments indicate that neither copper acetate nor acetylcholine is capable

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* Aided by a grant from the Committee for Research in Problems of Sex, National Research Council.

[1] Fevold, H. L., Hisaw, F. L., and Greep, R., *Am. J. Physiol.*, 1936, **117**, 68.

[2] Harris, G. W., *J. Physiol.*, 1941, **100**, 231.

[3] Brooks, C. M., Beadenkopf, W. G., and Bojar, Samuel, *Endocrinology*, 1940, **27**, 878.

[4] Taubenhaus, M., and Soskin, Samuel, *Endocrinology*, 1941, **29**, 958.

[5] Dey, F. L., *Am. J. Anat.*, 1941, **69**, 61.

[6] Brookhart, J. M., Dey, F. L., and Ranson, S. W., *Endocrinology*, 1941, **28**, 561.

of inducing the liberation of luteinizing hormone in the constant estrous guinea pig. The closure of the vaginal membrane in the one animal injected with copper acetate and the partial closure in the 4 animals injected with acetylcholine was probably due to the debilitating effects of the injections rather than to a specific action of the drugs since no evidence of ovulation nor of luteinization could be found in the ovaries.

Summary. Lesions of the anterior hypothalamus of the female guinea pig may prevent the secretion of luteinizing hormone. The ovaries of these animals undergo marked follicular development but ovulation and luteinization fail to occur. Injections of copper acetate and of acetylcholine failed to induce the liberation of luteinizing hormone in these animals although several routes of injection were employed.

14136 P

Treatment of Circulatory Collapse of Experimental Venous Occlusion: Use of Adrenal Cortical Extract and Saline Solution.

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The appearance of a shock-like syndrome and a high mortality rate has been demonstrated in untreated dogs subjected to occlusion of the venous drainage in one leg.[1,2] It has been reported, moreover,[3] that pre-treatment with desoxycorticosterone acetate (DCA) or with adrenal cortical extract (ACE) lengthened the survival time and, in a number of cases, resulted in complete recovery. Postoperative treatment with DCA, so far as recovery was concerned, proved relatively ineffective.[4]

The crucial problem, however, in an experimental attack upon the control of shock would appear to lie, not in the pre-treatment of incipient shock-like states, but in the contravention of such crises once they have set in. The present report deals with the postoperative use of ACE and of physiological saline, administered only after unmistakable signs of collapse have appeared.

Methods. Animals subjected to venous occlusion in one hind limb by suitable ligation and by injection of lampblack,[2] under pentobarbital anesthesia, were followed during their postoperative course for signs of a failing circulation and of systemic distress; mean arterial pressure (femoral puncture), respiratory rate, pulse, and rectal temperature were recorded at intervals. Symptoms of prostration appeared within a postoperative period ranging from 2 to 8.5 hours (Table I); the mean arterial pressure, for example, had fallen to levels as low as 27 mm Hg., and several of the animals were comatose. The therapy then instituted consisted of intravenous administration of ACE (Eschatin*), 3 cc per kg body weight, supplemented by subcutaneous injection of physiological saline, 4% of body weight.

Results. In a series of 18 experiments there were significant increases in the survival time in 6 animals (Table I, animals 2, 3, 5, 6, 8, 10). Of these, 4 were sacrificed after a 2-3-day survival period, at which time they were in good condition. Although no actual measurements were made, the legs with venous drainage occluded appeared to have undergone some reduction in size. Animal 10 was a complete survival, and 3 weeks after operation was in excellent condition; the limb with venous occlusion had returned to normal size. The survival times

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[1] Perlow, S., Killian, S. T., Katz, L. N., and Asher, R., *Am. J. Physiol.*, 1941, **134**, 755.

[2] Bourque, J. E., and Kotalik, G. C., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 371.

[3] Katz, L. N., Killian, S. T., Asher, R., and Perlow, S., *Am. J. Physiol.*, 1942, **137**, 79.

[4] Katz, L. N., Asher, R., and Perlow, S., *Proc. Cent. Soc. Clin. Res.*, 1942, **15**, 36.

* We are grateful to Dr. Oliver Kamm of Parke, Davis and Company, for generous supplies of Eschatin.

TABLE I.
Effect of Adrenal Cortical Extract and Physiological Saline upon Survival Time.

Dog	Wgt	Inj. time p.o. (hr)	Mean art. pressure (mm Hg.)	Wt incr. of limb (% body wt)	Survival time (hr)
1	5.7	2.3		6.1	9.0
2	5.2	2.5		2.3†	72.0*
3	6.8	2.08		2.3†	72.0*
4	6.4	3.08	80	1.9	16.2
5	4.1	5.3	53	1.9†	48.0*
6	5.5	8.5	27	4.0†	48.0*
7	7.3	4.0	60	3.9	10.0
8	5.5	2.8	40		72.0
9	3.2	2.8		3.1	21.8
10	5.5	4.4			complete
11	7.3	4.9		3.7	10.0
12	4.1	3.4			10.5
13	4.3	3.9			9.2
14	4.6	4.9	50		23.0
15	4.6	6.0	42		16.75
16	5.5	4.9	32		7.9
17	5.5	2.0			5.8
18	6.4	2.0			9.5

*Sacrificed. †At sacrifice.

of the remaining 12 ranged from 5 hours 45 minutes to 21 hours 50 minutes. In 12 control experiments on untreated animals[2] survival times ranged from 3 hours 45 minutes to 16 hours 45 minutes. In another series of 12 control experiments, ACE alone was injected intravenously (3 cc/kg), without the saline adjuvant. Survival times ranged from 4 hours to 18 hours. In a third control series, 0.9% sodium chloride solution was injected subcutaneously, 4% of body weight. Two animals survived 3 and 5 days respectively. The range of survival times of the remaining 10 ran from 4 hours 45 minutes to 38 hours 15 minutes.[†]

Data on femoral arterial pressure are inadequate for any judgment as yet regarding their validity as a criterion of collapse and for initiation of treatment. The range at the time of general prostration varied in the limited number observed in this preliminary series. In dog 6, for example, the mean pressure had fallen to 27 mm Hg.; in dog 4, on the other hand, clinically comparable, the pressure prior to treatment was 80 mm Hg. However, dog 6 survived and dog 4 died at the end of 16 hours 15 minutes. It seems highly probable that factors other than a failing circulation are involved in precipitation of irreversible collapse.

The high mortality of the experimental series—since two-thirds survived no longer than the controls, cannot be explained as yet. The dogs were in excellent health preoperatively and treatment was identical in each case; the operative technic, moreover, had been perfected in over 60 dogs prior to this series. The danger of obtaining fallacious values for any given course of therapy, and the need for extended observations, is indicated by the circumstance that the 6 animals with prolonged survival times were obtained in the first 8 animals of the series; had work ceased at that point, a definitely beneficial effect could have been reported in 75% of the animals. As it turns out, the treatment was effective in certain instances, but the nature of the precise determining factor remains to be determined.

Summary. The shocklike syndrome of circulatory collapse and prostration was induced in a series of 18 dogs by a regional venous occlusion. Treatment at the time of collapse, by infusion of ACE and physiological saline, resulted in a marked increase in survival time in 6. In a control group of 12, saline infusion alone prolonged survival time in 2. ACE alone in a series of 12 proved ineffective.

[†] Statistical analysis shows that difference in survival times of saline treatment and of extract treatment alone, are significant. Saline survivals were longer. No significant difference was found between untreated controls and Eschatin alone.

14137 P

Antigenic Relation of Type B *H. Influenzae* to Type 29 and Type 6 Pneumococci.*

H. D. ZEPP AND H. L. HODES. (Introduced by E. A. Park.)

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In January, 1942, infant R.B. was admitted to the hospital with purulent meningitis. *Hemophilus influenzæ* type B was isolated from the spinal fluid and type 6 pneumococcus from his nasopharynx. The child was treated with sulfadiazine and anti-*H. influenzæ* type B rabbit serum (Squibb), recovered completely and was discharged. He was re-admitted with symptoms of meningitis in May, 1942, when a type 6 pneumococcus was recovered from his spinal fluid and from his nasopharynx. Because the child had previously recovered from *H. influenzæ* meningitis, the type 6 pneumococcus from the spinal fluid was tested against type B anti-*H. influenzæ* horse serum supplied by the Massachusetts State Health Department. The quellung obtained with the *H. influenzæ* serum was quite definite but not so marked as that obtained with the type 6 pneumococcus serum. Conversely, it was found that a type B *H. influenzæ* organism recently isolated from the spinal fluid of another child quelled in the presence of type 6 anti-pneumococcus therapeutic rabbit serum (Lederle), although it did not do so when tested against type 6 anti-pneumococcus typing serum (Table I). We have since tested 3 other recently isolated type 6 pneumococci against type B anti-*H. influenzæ* horse and rabbit sera. Two of these organisms were quelled by these sera. We have also tested stock strains of pneumococci from Type 1 through 33 against anti-*H. influenzæ* type B rabbit and horse serum. It was found that in addition to type 6 pneumococcus, type 29 pneumococcus gave marked quellung in the presence of both these sera, but none of the other types tested have so reacted (Table I). We have since found that 3 other strains of type 29 pneumococcus recently isolated from the nasopharynx of children with pneumonia have also shown marked capsular swelling in the presence of anti-*H. influenzæ* type B horse and rabbit serum. In addition, we have found that type 29 pneumococcus rabbit therapeutic serum (Lederle) causes quellung of *H. influenzæ* type B. This reaction is more marked with the type 29 pneumococcus serum than it is with the type 6 serum (Table I). Seven strains of *H. influenzæ* isolated since May, 1942, have also exhibited marked capsular quellung in the presence of type 29 anti-pneumococcus therapeutic rabbit serum (Lederle), and to somewhat lesser degree also in the presence of type 6 anti-pneumococcus therapeutic rabbit serum (Lederle) (Table I).

We have also tested strains 62B and 191, type B *H. influenzæ,* furnished us by Dr. John Dingle and Dr. Leah Seidman. Both strains showed marked capsular swelling with type 29 anti-pneumococcus therapeutic rabbit serum, but gave only a doubtful quellung reaction with the type 6 anti-pneumococcus therapeutic rabbit serum.

Because of the rather remote possibility that the commercial sera which had been employed might have been derived from animals which had been inoculated at different times with both pneumococci and *H. influenzæ,* it was thought advisable to prepare sera in our own laboratory. Two rabbits were given a course of intravenous inoculations with 6-hour cultures of the 62B strain and 2 other animals received a similar course with the 191 organism. Both rabbits which had been inoculated with the 62B organisms yielded sera which caused very great capsular swelling of type 29 pneumococci. The serum of the 2 rabbits vaccinated with the 191 strain caused only very slight or negative capsular swelling with type 29 pneumococci. None of the 4 rabbit sera produced definite capsular swelling of

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TABLE I.

Quellung Reactions Obtained with Various Strains of Type 6 and Type 29 Pneumococci, and Type B *H. influenzæ* When Mixed with Various Commercially Prepared Sera.

Organism	Type	Type B *H. influenzæ* horse serum (Mass. State)	Type B *H. influenzæ* rabbit serum (Squibb)	Type 6 rabbit pneumococcus typing serum (Lederle)	Type 6 rabbit pneumococcus therap. serum (Lederle)	Type 29 rabbit pneumococcus typing serum (Lederle)	Type 29 rabbit pneumococcus therap. serum (Lederle)
Pneumo. (R.B.)	6	+ quellung	+ quellung	4+ quellung	4+ quellung	Neg.	Neg.
” (C.F.)	6	+ ”	+ ”	4+ ”	4+ ”	”	”
Pneumo. (Lederle)	6	+ ”	+ ”	4+ ”	4+ ”	”	”
Pneumo. (Sydenham)	6	— ”	— ”	4+ ”	4+ ”	”	”
Pneumo. (Lederle)	29	3+ ”	3+ ”	Neg.	Neg.	4+ quellung	4+ quellung
Pneumo. (Strain 1)	29	3+ ”	3+ ”	”	”	4+ ”	4+ ”
Pneumo. (Strain 2)	29	3+ ”	3+ ”	”	”	4+ ”	4+ ”
Pneumo. (Strain 3)	29	3+ ”	3+ ”	”	”	4+ ”	4+ ”
H. influen. (Strain 1)	B	4+ ”	4+ ”	”	+ quellung	Neg.	3+ quellung
H. influen. (Strain 2)	B	4+ ”	4+ ”	”	+ ”	”	3+ ”
H. influen. (Strain 3)	B	4+ ”	4+ ”	”	+ ”	”	3+ ”
H. influen. (Strain 4)	B	4+ ”	4+ ”	”	+ ”	”	3+ ”
H. influen. (Strain 5)	B	4+ ”	4+ ”	”	+ ”	”	3+ ”
H. influen. (Strain 6)	B	4+ ”	4+ ”	”	+ ”	”	3+ ”
H. influen. (Strain 7)	B	4+ ”	4+ ”	”	+ ”	”	3+ ”
H. influen. (Strain 62B)	B	4+ ”	4+ ”	”	± ”	”	3+ ”
H. influen. (Strain 191)	B	4+ ”	4+ ”	”	± ”	”	3+ ”

type 6 pneumococci. It should be noted that all of these sera were unconcentrated (Table II).

Rabbit sera were also prepared by us against type 6 and type 29 pneumococci. The 2 strains used had been obtained from Lederle Laboratories in May, 1941. One rabbit was given a course of intravenous inoculations with living type 6 pneumococci while another rabbit received heat-killed type 6 organisms. Similarly, 2 rabbits were inoculated with living and one with heat-killed type 29 pneumococci. As can be seen in Table II, the animal inoculated with living type 6 pneumococci yielded serum which caused very marked capsular quellung with the homologous organism but did not cause any capsular swelling of the type 29 pneumococcus. When tested against an 18-hour culture of recently isolated type B *H. influenzæ,* a limited but definite quellung of the capsule of this organism occurred. The serum obtained from the rabbit inoculated with heat-killed type 6 pneumococci caused capsular swelling of only the homologous pneumococci. The sera of the 2 rabbits vaccinated with living type 29 pneumococci did not cause quellung of the type 6 pneumococci. They did, however, produce marked quellung of both type 29 pneumococci and type B *H. influenzæ.* The serum of the rabbit vaccinated with heat-killed type 29 pneumococci caused marked quellung of type 29 pneumococci and a definite but much less marked reaction with type B *H. influenzæ.*

Discussion. It appears that the capsule of type B *H. influenzæ* has an antigen in common with the capsule of at least some strains of the type 29 and type 6 pneumococcus, but

TABLE II.
Quellung Reactions with Unconcentrated Rabbit Sera Prepared by Authors.

Rabbit serum No.	Vaccine used in preparation of serum	Culture used in quellung test		
		Type 6 pneumo.	Type 29 pneumo.	Type B *H. influenzæ*
1316	Living type B *H. infl.* (62B)	Neg.	2+ quellung	4+ quellung
1317	” ” ” ” ” ”	”	2+ ”	4+ ”
1318	” ” ” ” ” (191)	”	± ”	4+ ”
1319	” ” ” ” ” ”	”	Neg.	4+ ”
1386	” ” 6 pneumococci	4+ quellung	”	+ ”
1387	Heat killed type 6 pneumococci	4+ ”	”	Neg.
1388	Living type 29 pneumococci	Neg.	4+ quellung	4+ quellung
1389	” ” ” ”	”	4+ ”	4+ ”
1390	Heat killed type 29 pneumococci	”	4+ ”	+ ”

in the latter instance this relationship is much less clear cut. This and other evidence thus far accumulated indicates that the antigenic components involved in these cross reactions are not identical. The clarification of the relationship involved must await absorption experiments. Types of *H. influenzæ* other than type B are being investigated. It is very probable that other relationships between *H. influenzæ* and the pneumococcus group will be uncovered as they are sought. In fact, while this article was being prepared for publication, Chapman and Osborne[1] stated that they had discovered a mutual quellung reaction between pneumococcus type 6B and *H. influenzæ* type A. These authors also described the quellung of *H. influenzæ* type C in type 21 anti-pneumococcus rabbit serum.

Conclusion. Four strains of type 6 pneumococci and 4 strains of type 29 pneumococci have been encountered which appear to have one or more capsular antigens in common with 7 strains of type B *H. influenzæ* which have recently beeen recovered from the spinal fluid or blood of infants. This relationship has been demonstrated by the capsular quellung method.

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[1] Chapman, O. D., and Osborne, W., *J. Bact.*, 1942, **44**, 620.

14138

Effect of Thymoxyethyldiethylamine on Various Pain Thresholds with Special Reference to Referred Pain.*

Sol Roy Rosenthal, David Minard and Edward Lambert.

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Observations that the phenol ether, thymoxyethyldiethylamine, administered to unanesthetized dogs produced a generalized elevation of the cutaneous threshold for pain stimuli without evident signs of central narcosis[1] suggested to the authors its possible utility in the experimental study of visceral pain.

Method. Adult untrained dogs were used. The threshold for pain was determined by electrical stimuli supplied from a Harvard inductorium with 4.5 v on the primary circuit. Faradic stimuli of graded intensity were applied by means of bipolar electrodes in contact with the shaved abdominal skin, the parietal and visceral peritoneum of the abdomen, me-

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* Aided partially by a grant from Abbott Laboratories.

[1] Rosenthal, S. R., and Minard D., *J. Exp. Med.*, 1939, **70**, 415.

senteric nerve trunks, and the exposed saphenous nerve. The pupillodilator reflex served as a reliable index of pain responses. The electrical threshold for eliciting this reflex was expressed as the distance in centimeters of the secondary from the primary coil, this distance being inversely related to the intensity of the stimulus.

The drug was administered in 1 or 2% solution of the hydrochloride in doses ranging from 30 to 50 mg per kg expressed as the base either parenterally by subcutaneous injection or enterally by rectum or by mouth. In the latter case atropin sulfate (.05 mg per kg) was injected 15 minutes before giving the drug to allay drug loss from vomiting.

To insure more uniform results all extraneous stimuli tending to disturb the animal, such as loud talking, barking of other dogs, and rough handling must be strictly avoided during the initial stages of the drug action. Later, such precautions are less essential.

In the first experiment (dog No. 1) control values for the various thresholds were obtained before administering the drug, the abdomen being opened under local (procaine) anesthesia. In subsequent experiments opening of the abdomen and visceral stimulation was postponed until the drug had taken maximal effect as indicated by the elevation of the skin threshold. In some experiments readings were then taken periodically until the pain thresholds of viscera and skin had again approached normal. In 5 of the 27 experiments in which the edges of the abdominal wound were infiltrated with solutions of procaine or of the drug to eliminate a possible decrease in sensory threshold at these sites, a further elevation of skin and visceral thresholds was noted.

Results. The thymoxyethyldiethylamine preparations used in these experiments were effective in producing an elevation of the cutaneous threshold in approximately 80% of the dogs tested. Its failure to act in some animals has not been investigated.

The experimental results in a series of 27 dogs in which definite cutaneous analgesia was obtained are given in the accompanying tables. The values given represent the setting of the secondary coil at threshold intensity.

For consideration the experiments are divided into two groups, the first (Table I) including dogs in which the skin threshold following administration of the drug increased 3.5 cm or more, and the second group (Table II) those in which the increase was 3 cm or less. Stimuli with a secondary coil setting of less than 2 cm from the primary were avoided to prevent tissue damage.

In 7 of 9 dogs in the first group the threshold obtained for the visceral peritoneum covering the free border of the small intestine was found to be 2 cm or higher during the maximal drug effect. In the second group this degree of threshold elevation was found in only 6 of 15 animals. In all experiments in which readings were taken repeatedly, the threshold values for both skin and viscera decreased gradually as the drug action diminished. In general, values obtained for parietal and for visceral peritoneum varied in the same direction.

Similarly the mesenteric nerve threshold was found to be elevated to a greater extent in the first group than in the second; in general, the changes in threshold of the mesenteric nerve approximately paralleled the threshold changes in skin and peritoneum.

In 9 experiments in which the threshold values for the saphenous nerve were determined both before and after drug administration, little or no change was evident. However, determinations with an instrument giving absolute values of current intensity would be desirable since intensities in the range used in these experiments were not comparable on an absolute scale.

In one experiment (dog No. 12) sodium pentobarbital (20 mg per kg) was given with the thymoxyethyldiethylamine. The saphenous nerve threshold, taken during the light general anesthesia which ensued, was elevated to 3 cm.

Discussion. Thymoxyethyldiethylamine administered enterally or parenterally induces a reversible generalized cutaneous analgesia in the majority of the dogs tested. Although not definitely established the site of this action of the drug is believed to be peripheral since little or no change in electrical threshold of the saphenous nerve, a somatic sensory trunk, could be detected by the method employed.

TABLE I.

Various Electrical Thresholds, as Determined by the Harvard Inductorium, Before and After the Administration of Thymoxyethyldiethylamine. (Elevation of skin threshold of 3.5 cm or more.)

	Skin threshold, cm		Serosa intestine, cm		Mesenteric nerves, cm		Saphenous nerve trunk, cm	
Dog No.	Before	After	Highest Reading	Lowest Reading	Highest Reading	Lowest Reading	Before	After
1	6	2.5	8.5‡	2§	10‡	4.5	13 -80°	13 -80°
2	6.5	2		2		2	12 -30	12 -30
3	6.75	2.5		2.5	10	4	12.5-70	12.5-60
4	6.25	2	5	2	5.5	2.9	12.5-30	12.5-30
5	6.25	2		2	6	2	12.5-60	12.5-60
6	6.25	2		2	7	5		
7	6.25	2		6.5		8		
8	5	1	8	6			12 -80	12 -80
9	6	2				3		
10	6.75	2		2		5.5		
11	7	2						12 -85†
12	6.5							3*

*Pentobarbital sodium and thymoxyethyldiethylamine injected.
†Abdomen not opened.
‡Readings taken before injection of thymoxyethyldiethylamine.
§Abdominal incision injected.

TABLE II.

Various Electrical Thresholds, as Determined by the Harvard Inductorium Before and After the Administration of Thymoxyethyldiethylamine. (Elevation of skin threshold of 3 cm or less.)

	Skin threshold, cm		Serosa intestine, cm		Mesenteric nerves, cm		Saphenous nerve trunk, cm	
Dog No.	Before	After	Highest Reading	Lowest Reading	Highest Reading	Lowest Reading	Before	After
13	5.75	4.5	8	2	10.5	6	12 -80°	12 -80°
14	6.75	4		2		5		12 -45
15	6.75	5.5		7.5		10		
16	6.5	4.75		1*		5.5	13 -70	
17	6.25	5	10	3*	10	6	13 -60	13 -60
18	6.5	4	8	3*	10	3		12.5-30
19	5.75	3.25		2.5	6	4.25	12 -70	12 -40
20	6.25	3.5	7	3*	8	3		12 -30
21	6.5	4	6	3		6.5		13 -30
22	7	5		2		2		11 -65
23	6.75	3		3		3		12.5-30
24	6	4		6.75		6.75		11 -60
25	6.5	3		1		1		12 -85
26	6.25	3.5	6	2	7	5		
27	6	3.25		2		2		

*Abdominal incision injected.

Climenko, Homburger and Messer[2] in a recent study of the pharmacology of this drug noted a local anesthetic action at the site of injection which they ascribe to local tissue injury but these authors fail to discuss the generalized sensory effect previously noted by two of us.[1]

Burchell and Varco,[3] in a study of the effect of the drug on gastric secretion in dogs, report a lowered sensitivity to pain in 4 animals but only when the drug was given in doses of 75 mg per kg. The discrepancy in dosage and degree of action between these findings and our own requires further investigation. More recent experiments in which the variability in action of newer preparations of the drug was more marked than in the earlier experiments reported here suggest that the potency of different preparations may vary.

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[2] Climenko, D. R., Homburger, E., and Messer, F. H., *J. Lab. and Clin. Med.*, 1941, **27**, 289.

[3] Burchell, H. B., and Varco, R. L., *J. Pharm. and Exp. Therap.*, 1942, **75**, 1.

In contrast to its lack of effect on the threshold of somatic nerve, the drug produces an elevation of the sensory threshold of viscera and visceral nerve which is roughly proportional to the change in skin threshold. Unless one postulates an action of the drug on visceral sensory nerve differing in effect from that on somatic sensory nerve, the findings reported here support the view that in the dog visceral pain is referred wholly or in part to the skin.[4]

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[4] Rosenthal, S. R., and Lambert, E. H., in publication.

14139

Physiological Effects at Low Oxygen Tensions of Replacing Oxygen with Carbon dioxide.*

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The following experiments were designed to determine if, on the basis of increased respiratory minute volume, an increased alveolar oxygen tension may be obtained at low oxygen tensions by replacing some of the oxygen with an equal amount of carbon dioxide. Johnson, Eckman, Ramsay and Barach,[1] in experiments on nembutalized dogs in a low pressure chamber, found that addition of 14.3 mm Hg of CO_2 produced no beneficial effect on the oxygen tension of mixed venous blood. The value of these results is limited by the fact that nembutalized dogs are not normally sensitive to the stimulatory effects of carbon dioxide on respiration. However, in a subsequent study which included 4 human subjects, Himwich, Fazekas, Herrlich, Johnson and Barach[2] found that the oxygen content of the arterial and cerebral venous blood was approximately the same whether a 10% CO_2-90% O_2 mixture or 100% O_2 was breathed at a simulated altitude of about 35,000 feet. Numerous studies have demonstrated that addition of CO_2, without proportionate removal of oxygen, has a beneficial effect when the original alveolar oxygen tension is below that required for normal oxygenation of hemoglobin. However, the problem in the latter case is quite different than when the sum of the oxygen tension plus the carbon dioxide tension is kept constant.

Procedures. Eighteen men between the ages of 23 and 35 were used as subjects. These subjects had no known respiratory or cardiovascular abnormalities. The procedure for performing an experiment was as follows. The subject lay on a cot in the supine position for 10 minutes. Then a sample of alveolar air was obtained by collecting the last 200 cc of a forced expiration begun at the end of a normal expiration, and the oxygen and carbon dioxide content of the sample was determined immediately by the use of the Haldane gas analysis apparatus. After allowing the subject a few minutes to equilibrate, following collection of the alveolar sample, he was connected by means of a mouthpiece with a spirometer containing air at 23 to 25°C and saturated with water vapor and at a pressure of 743 to 755 mm Hg. Two one-way valves placed close to the mouthpiece were so arranged that the subject inhaled from the spirometer and exhaled through a gas meter offering a

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* Aided by a grant from the John and Mary R. Markle Foundation.

[1] Johnson, A. E., Eckman, M., Ramsey, C., Jr., and Barach, A. L., *J. Aviation Med.*, 1942, **13**, 130.

[2] Himwich, H., Fazekas, J., Herrlich, H., Johnson, A. E., Barach, A. L., *J. Aviation Med.*, 1942, **13**, 177.

TABLE I.

Comparison of Respiratory and Cardiovascular Effects of Oxygen-Carbon-dioxide-Nitrogen Mixtures.

Composition of gas mixtures	Avg alveolar CO_2 after breathing mixture for 8 minutes	Alveolar O_2 after breathing mixture for 8 minutes*	Blood pressure changes	Heart rate incr., beats/min.*	% incr., R.M.V.*	Alveolar O_2 tension (calc.) % sat. of Hb. (calc.)	
						mm Hg	% sat.
10-2-88	5.6	5.5-6.7	No change 6; remainder, systolic 4-12 mm rise; diastolic no rise.	10-22	20-100	45	80
12-0-88	5.5	5.6-6.8	No change 9; remainder, systolic 4-10 mm rise; diastolic no rise.	10-18	2-29	46.5	81
12-2-86	5.8	6.8-7.8	No change 4; remainder, systolic 4-10 mm rise; diastolic 4-10 mm rise one-half of cases.	4-13	29-65	54.8	87.5
12-3-85	5.9	7.9-9.5	No change 2; in 16 cases 4-10 mm rise systolic; milder rise diastolic.	4-15	53-101	65.25	90

* Range includes middle 16 of 18 experiments.

resistance of less than 5 mm of water. Rebreathing was negligible as indicated by the fact that the same reading for respiratory minute volume was obtained when the subject inhaled from the spirometer and exhaled through the gas meter as when he inhaled room air through the nose and exhaled from the mouth through the gas meter. Moreover, the dead space remained constant throughout the experiments. A mask was not used because the best fitting masks contained sufficient dead space to cause a significant increase in respiratory minute volume over that obtained when a mouthpiece was used. After the subject was connected to the apparatus for 3 or 4 minutes, recording of blood pressure, heart rate, and respiratory minute volume was begun. Within 4 to 8 minutes after constant figures for these determinations were obtained one of 4 gas mixtures contained in cylinders was fed through a humidifier and temperature regulator into the spirometer instead of air. Recording of respiratory minute volume and heart rate and blood pressure was continued until a few minutes after they became constant. This occurred in all cases within 8 minutes. Another alveolar air sample was obtained at the end of the period of breathing the gas mixture.

Results. The effects of each of the four gas mixtures on the 18 subjects are summarized in Table I. It may be noted that there is no significant difference in the alveolar oxygen tension after breathing the 12-0-88 mixture as compared with that after breathing the 10-2-88 mixture. The increased respiratory minute volume produced by the later is barely sufficient to compensate for its lower oxygen tension. The heart rate and blood pressure increase similarly with the two mixtures. The rise in heart rate with no decrease in pulse pressure is compatible with Grollman's[3] finding that an increased cardiac output occurs when the percent saturation of hemoglobin of arterial blood falls below 85. It is possible that the fraction of the total cardiac output reaching the brain is not the same when breathing the two different mixtures; however, the experiments of Himwich *et al.*[2] indicated no increase in the oxygen tension of the cerebral venous blood.

The wide individual differences in sensitivity of the respiratory mechanism to the stimulatory action of oxygen-lack and carbon dioxide excess is shown by the figures indicating the percentage increase in respiratory minute volume. There is also a considerable variation in the cardio-accelerator response to oxygen lack, but the 12-0-88 mixture produced a significant increase in heart rate in all cases while a significant increase in respiratory minute volume (greater than 6%) occurred in only 14 of the 18 cases.

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[3] Grollman, A., *Cardiac Output in Man in Health and Disease*, Springfield, Ill., Chas. C. Thomas, 1932.

The only significant difference in response to the 10-2-88 mixture as compared with the response to the 12-0-88 mixture was the greater respiratory activity produced by the former. Therefore, the results indicate that no beneficial effects would be obtained by substituting carbon dioxide for oxygen when the original oxygen tension is insufficient for normal saturation of the hemoglobin of arterial blood. On the other hand, it is apparent that a considerable part of the oxygen in inspired air may be replaced with carbon dioxide without a reduction in alveolar oxygen tension.

Comparison of the effects of the last 3 mixtures listed in Table I indicates that increasing the carbon dioxide in the inspired air, when the oxygen tension of the inspired air is deficient but is kept constant, results in an increase in the alveolar oxygen tension on the basis of increased respiratory minute volume. This result is in accord with a number of observations reported in the literature of relief of hypoxia obtained by increasing the carbon dioxide tension of inspired air without a corresponding reduction of oxygen tension. However, the alveolar oxygen tension when breathing the 12-2-86 and 12-3-85 mixtures is not significantly higher in the average case than the alveolar oxygen tension that would be expected to result from breathing 14% and 15% oxygen respectively even if no respiratory stimulation occurred. This does not necessarily mean that the oxygen tension in tissues would be the same in each case.

Comment. In view of the above results, in the presence of a degree of hypoxia that will allow a "leveling off" of the blood pressure and heart rate and respiratory minute volume within a few minutes after the onset of the hypoxia, it appears unlikely that increasing the carbon dioxide tension of the inspired air will produce any beneficial results beyond that produced by addition of an equivalent oxygen tension. It is possible that a different result would be obtained in the presence of more severe hypoxia, but such a degree of hypoxia would be tolerable for only a brief time.

14140

The Vasodilator Properties of a Pancreatic Extract.*

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Since the demonstration by Frey and Kraut[1] of the presence of a depressor substance in urine and blood and the subsequent preparation of extracts of pancreas with similar effects upon blood pressure, many clinical trials have been made with pancreatic extracts. Most of the reports deal with the so-called circulatory hormone of the pancreas, kallikrein or "Padutin" (see Rigler[2] for review of literature). A deproteinated extract of pancreas,* free of insulin, choline esters, and histamine, has been tried with reported success in various clinical conditions. In many of the clinical studies, however, no convincing control observations were made, and the subjective response of the patient was often the accepted criterion.

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* The pancreatic extract used is manufactured and supplied by Sharp & Dohme, Inc., under their trade name, "Depropanex" deproteinated pancreatic extract. It is stated to contain not more than 2.5% solids, including not more than 0.5% nitrogen, 0.9% sodium chloride, and 0.25% phenol as a preservative. This investigation was aided by a grant from Sharp & Dohme, through the courtesy of Dr. L. Earle Arnow.

† Present address: Mayo Aero-Medical Unit, Rochester, Minn.

[1] Frey, E. K., and Kraut, H., *Z. physiol. Chem.*, 1926, **157**, 32.

[2] Rigler, R., *Heffter's Handb. exp. Pharmakol.*, 1938, **7**, 63.

Elliot and Nuzum,[3] comparing an insulin-free pancreatic extract with "Padutin," found that intravenous doses of either preparation in rabbits caused a brief fall in blood pressure and that repeated doses led to a more prolonged hypotension. In hypertensive patients intravenous administration of the extracts caused a fall in systolic pressure lasting as long as an hour; but no effect was observed on the blood pressure of normal human subjects and no effect was found following subcutaneous or intramuscular administration in either rabbits or human subjects. The ineffectiveness of intramuscular and oral administration to animals of large doses of an insulin-free pancreatic extract was also emphasized by Van Winkle.[4]

Since no studies had been made of the effect of pancreatic extract on blood flow, with the exception of the Langendorff perfusion experiments of Elliot and Nuzum, it seemed desirable to record the action of this material upon coronary blood flow in the dog heart-lung preparation and upon femoral arterial flow in cats in an attempt to establish whether or not there is any experimental basis for its clinical use.

I. *The effect of pancreatic extract upon coronary sinus outflow in the denervated and innervated heart-lung preparation. Methods.* Studies on the coronary circulation were carried out on the denervated heart-lung preparation of the dog. Dogs of both sexes, weighing 10 to 15 kg, were used under "Nembutal" or chloralose anesthesia. Defibrinated blood obtained from an etherized bleeder dog was used. Coronary sinus outflow was measured by means of a Morawitz cannula and a Condon flow recorder. Aortic pressure was recorded by means of a mercury manometer and was kept constant in all experiments.

Several experiments were performed with the innervated heart-lung preparation in which the head was perfused from a pump-oxygenator system; no vascular connection existed between the head and thorax.

Results. In 4 experiments single doses of pancreatic extract, resulting in concentrations‡ of from 1:1000 to 1:700 in the coronary blood, exerted no effect upon coronary flow. Great but transient increases in flow resulted when the extract was administered in concentrations of 1:200 to 1:100.

In one experiment an attempt was made to determine the minimum effective concentration. Pancreatic extract was injected into the inflow tubing from a constant rate infusion pump. As shown in Fig. 1, the initial sinus flow was 19 cc per minute. An initial dose of 1.0 cc injected rapidly into the inflow tubing almost doubled the rate of blood flow, but for less than a minute. A control injection of 1.0 cc of 0.25% phenol (used as a preservative in the pancreatic extract) failed to produce any dilatation. Following this, pancreatic extract was infused at a rate of 0.66 cc per min for 4¼ minutes, a total dose of 2.84 cc. The total output of the heart was about 440 cc per minute; the concentration in the inflowing blood was therefore 1:660, assuming no cumulation in the blood. A negligible increase in coronary flow, less than 5%, occurred. The injection rate was then increased to 1.33 cc per minute, a concentra-

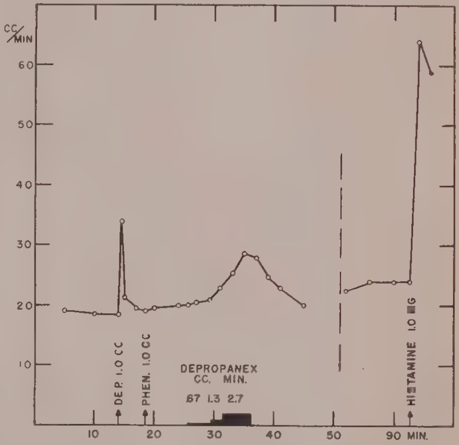

FIG. 1.

Coronary sinus outflow in the denervated dog heart-lung preparation. Injections directly into the venous inflow tubing. Arrow marked "Phen" signifies the injection of 0.25% phenol in saline.

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[3] Elliot, A. H., and Nuzum, F. R., *J. Pharmacol. Exp. Therap.*, 1931, **43**, 463.

[4] Van Winkle, W., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 220.

‡ Concentrations are stated in this report in terms of the volume of the pancreatic extract.

tion of 1:330, and the infusion continued for 2¼ minutes. As only a moderate increase in flow occurred, the injection rate was increased to 2.66 cc per minute, at a concentration of 1:165. The coronary flow increased gradually during the 4-minute infusion period to 29 cc per minute, an increase of 45% over the basal level. The flow returned to the control level in 9 minutes after the infusion was stopped.

Since coronary flow increased gradually during the infusion period, it is obvious that cumulation in the blood occurred; that is, the active substance was not destroyed by one passage through heart and lungs. However, in spite of a total dose of 18 cc (concentration of 2.7% in the entire blood volume) the flow rapidly returned to normal, indicating a rapid rate of inactivation or destruction. From this experiment it would appear that the minimal effective concentration in the denervated dog heart-lung preparation lies between 1:660 and 1:330, approximately 0.2%.

Later in this experiment 0.25% phenol in saline was infused for 4 minutes at 2.66 cc per minute without effect upon coronary flow.

In the experiments with the innervated heart-lung preparation comparable results were obtained. Pancreatic extract injected into the cardiac circulation in high concentration caused coronary dilatation, but no effects followed the injection of the extract into the cephalic blood supply, indicating that central or reflex factors are not concerned in the coronary dilator action of the substance.

II. *The effect of pancreatic extract upon peripheral blood flow. Methods.* Experiments on femoral arterial flow were performed on cats under ether, "Nembutal," or chloralose anesthesia; a stable control period was most regularly obtained with chloralose. The animals were heparinized (5 mg/kg) and cannulas inserted into the left carotid artery and the right external jugular vein for the recording of mean arterial pressure and the intravenous administration of drugs. The flow-meter was inserted into the right femoral artery by means of centrally and peripherally directed cannulas. The flow-meter was a coil of glass tubing graduated at 2 and 4 cc and contained in a constant-temperature bath. The rate of passage of a bubble of air large enough to occupy the whole diameter of the tube was timed with a stop-watch between the graduations. An air-trap at the top of the instrument collected the bubbles. Readings could be taken with an intervening time of only 2 or 3 seconds and were repeated as often as possible when changes were expected. From 5 to 10 minutes were required for insertion of the bubble stromuhr, but, since no attempt was made to interrupt collateral circulation, the leg was not subjected to severe asphyxia, and the flow soon reached a stable level. Observations were also made of cephalic blood flow in the innervated heart-lung preparation.

Results. The effect of pancreatic extract upon the femoral arterial flow was studied in 4 experiments. Doses of from 0.005 to 0.02 cc of the extract made up to 0.1 cc in physiological saline were injected into the peripheral limb of the stromuhr directly above the cannula. Numerous control injections of saline and of 0.25% phenol in saline proved to be without significant action. No attempt was made to determine the limiting concentration of pancreatic extract; at the basal level of blood flow in the various experiments the doses used reached the vessels of the leg in concentrations of from 3 to 14% in the blood, assuming no dilution from collateral channels. Representative results are illustrated in Fig. 2. Great but transient increases in blood flow occurred in every case, increases ranging from 118% to 650% of the control level. Blood flow in each instance returned to normal within 3 minutes after the injection.

Comparison was made of the pancreatic extract activity with that of glyceryl trinitrate, histamine, and acetylcholine. Pancreatic extract, 0.01 cc, was found to be roughly equivalent to 0.02 to 0.04 mg of glyceryl trinitrate; to 0.001 mg of histamine hydrochloride; or to 0.05 μg of acetylcholine chloride. Atropine in doses sufficient to abolish the effect of 1 μg of acetylcholine did not reduce the response to pancreatic extract.

Pancreatic extract in doses of 0.005 to 0.01 cc intravenously was found to have little or no action upon blood pressure or femoral flow

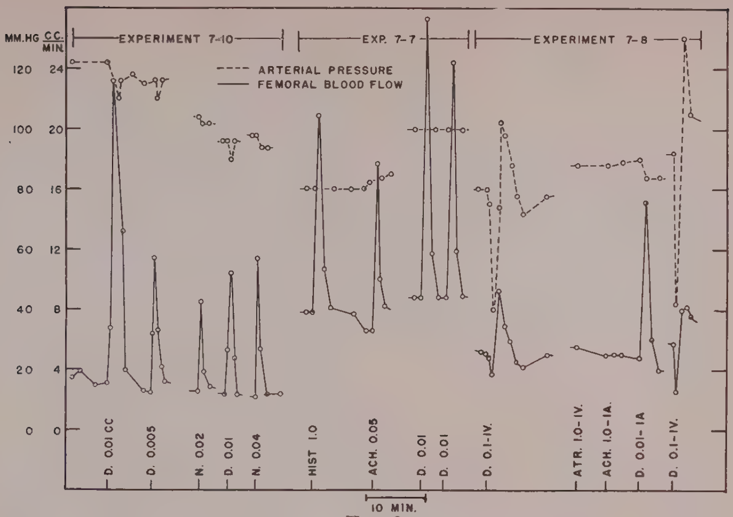

FIG. 2.

Arterial pressure and femoral arterial flow in anesthetized cats. D = pancreatic extract (dose in cc); N = glyceryl trinitrate (dose in mg); Hist. = histamine hydrochloride (dose in μg); Ach. = acetylcholine chloride (dose in μg; Atr. = atropine sulfate (dose in mg). All doses intra-arterial except those designated I.V. in the third experiment, which were intravenous.

in these cats, but in doses of 0.1 cc a profound fall in blood pressure always occurred. Mean arterial pressure fell from 80 to 42, from 80 to 40, from 92 to 42, and from 100 to 54 mm of Hg in 4 trials. The blood pressure decline lasted only one or 2 minutes and was followed by a rise of from 20 to 40 mm of Hg above the basal level, returning to normal within 4 or 5 minutes. Femoral flow after intravenous injection appeared to follow passively the changes in blood pressure. If vasodilatation did occur in the leg vessels, in no instance was it of sufficient degree to disturb significantly the positive correlation which existed between the blood pressure and femoral blood flow. If the blood pressure fall was caused by the vasodilating effect of the drug, it appears that the greater effect of the extract was exerted upon the splanchnic bed.

In the experiments upon the innervated heart-lung preparation the head was perfused through the brachiocephalic artery from a constant-output pump; changes in head perfusion pressure therefore reflected changes in vessel caliber in the head. Pancreatic extract, 1.0 cc, injected directly into the arterial stream, caused a transient but significant decrease in the perfusion pressure, indicating a dilatation of the cephalic arterial tree. In experiments in which the head perfusion pressure was kept constant and blood flow measured by means of a Weese stromuhr, 1.0 cc of pancreatic extract intra-arterially resulted in a transient increase in head blood flow. Concentrations were of the order of 1:400 to 1:200.

Conclusions. Although these experiments demonstrate that pancreatic extract exerts a vasodilator action upon the coronary, cephalic, and femoral arterial beds, the concentrations required are high (from 0.2 to 1%) and the duration of action very brief. The effects of large doses last, at most, 5 minutes. It is doubtful whether any increase in coronary flow would be observed with practicable doses in the intact animal; in the heart-lung preparation the aortic pressure is artificially maintained at a constant level, whereas the fall in blood pressure occurring in the intact animal would certainly decrease the magnitude of the coronary flow response. No evidence was found for any reflex change of coronary flow in the innervated heart-lung preparation. While no measurements of leg blood flow have been reported in human patients with peripheral vascular disease, it is doubtful that the recommended intramuscular doses could cause any change in flow in normal subjects.

Summary. 1. Pancreatic extract in concentrations of 0.2 to 1% causes a transient but significant increase in coronary sinus outflow in the denervated and innervated heart-lung preparation. 2. Femoral blood flow in anesthetized cats may be increased by 100 to 650% following doses of 0.005 to 0.02 cc of pancreatic extract injected intra-arterially. The effect lasts no more than 2 or 3 minutes. 3. Doses of 1 cc of pancreatic extract cause a transient dilatation of the arterial bed of the perfused dog head. 4. Doses of 0.01 cc have no regular effect upon arterial pressure when injected intravenously into anesthetized cats. Doses of 0.1 cc cause a 50% fall in arterial pressure lasting only one or 2 minutes, followed by a rise of 25 to 30% above the control level lasting for 3 or 4 minutes. Femoral blood flow appears to follow passively the arterial pressure; vasodilatation probably occurs chiefly in the visceral vascular beds.

The authors are indebted to Professor Otto Krayer for his aid and advice in the preparation of this report.

14141 P

Repair of Peripheral Nerves by Grafts of Frozen-Dried Nerve.*

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Orientation, growth rate and grouping of growing nerve fibers are determined by the biophysical and biochemical constitution of the interfaces along which they advance.[1] Their most favorable growth medium is degenerated peripheral nerve.[2] Accordingly, peripheral nerve fragments are the best means of bridging traumatic nerve gaps.[3] In mammals, autografts can be fully, homografts adequately successful;[4] heterografts are largely discredited.[4,5] Nerve grafting in man has met with the difficulties of (1) growth-obstructing fibrosis of the distal suture line, and (2) unavailability of grafts. The former may be obviated by resection of the barrier,[6] or possibly completely avoided if sutureless nerve splicing by arterial sleeves[7] should prove as successful in man as it has been in animals. The supply difficulty is more serious, since commonly no source other than the patient's own intact nerves is available. In order to avoid having to sacrifice a "minor" nerve for the repair of a more vital one, several authors have tried preserved or fixed nerves. Storage in petrolatum[8] leaves the grafts viable, but presumably not indefinitely. Alcohol-fixed grafts[8,9] lead to poor regeneration,[5] formalin-fixed grafts to complete failure.[9]

Basically, these past failures are attributable to the denaturation of the grafts. Therefore, one of us (P. W.) thought of trying a preserva-

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[2] Young, J. Z., *Physiol. Rev.*, 1942, **22**, 318.

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[5] Sanders, F. K., *Brain*, 1942, **65**, 281.

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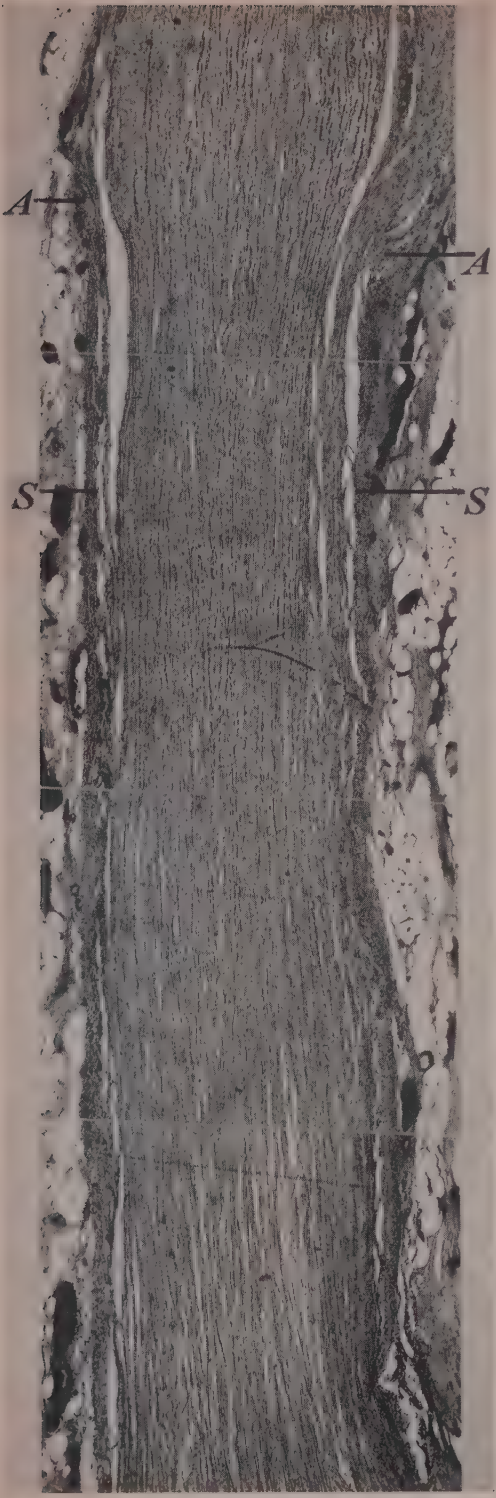

FIG. 1.
Regeneration through frozen-dried-rehydrated nerve graft sleeve-spliced to proximal stump of tibial nerve in rat, 127 days after operation. A, proximal end of arterial splicing sleeve. S-S, original level of fusion of proximal stump and graft.

tive, but non-denaturing treatment: the Altmann-Gersh freezing-drying method[10] used with great success in histology and histochemistry.[11] This method preserves microstructure and major biochemical (*e.g.*, enzyme) constitution without essential alteration. This very feature determines its suitability for the preparation of nerve grafts.

Our procedure is as follows: Nerves dissected aseptically are dropped into Isopentane[12] immersed in liquid nitrogen (–195°C), where they freeze instantaneously, and transferred to high vacuum and mercury pumps for about one week of dehydration over P_2O_5 at –40°C. The dry specimens are stored in sealed sterile containers. Before use, they are rehydrated in vapor at –40°C or in Ringer's solution *in vacuo* at room temperature. They resume their normal appearance and major histological characteristics,[13] including specific staining reaction (silver impregnation and Mallory Triple Azan).

Segments of these "devitalized" nerves of about 1-2 cm length were grafted into gaps of hind limb nerves of 38 rats, 4 cats, and 18 monkeys. These grafts were spliced to the nerve stumps by 2 short arterial sleeves,[7] or a single long sleeve containing the graft. Twenty-nine rat nerves (21 homoplastic, 8 cat-to-rat) have thus far been studied microscopically, and a few oscillographically, from 6 days to 18 weeks after the operation.

Homoplastic grafts heal and promote regeneration much as does live nerve. The regenerating sheath cells and nerve fibers invade them promptly, traveling in straight parallel courses, without appreciable branching or confusion. Fig. 1 shows a typical case, 4 months after grafting: regeneration is so perfect that there is no evidence of the old proximal "suture line" (level marked S-S). The regenerated fibers regain caliber (Fig. 2), impulse conduction is restored, and motility and sensitivity return. Nerves predegenerated

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[11] Bensley, R. R., and Gersh, I., *Anat. Rec.*, 1933, **57**, 205.

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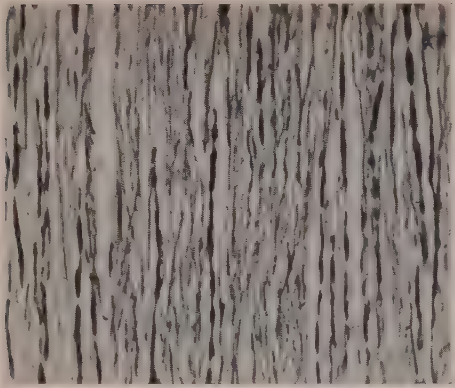

FIG. 2.
Detail from Fig. 1, showing the regenerated nerve in the zone of the graft. × 230.

before treatment do not seem superior to undegenerated ones. Details will be reported later. Sclerotic islands present in a few grafts may tentatively be ascribed to imperfections in the freezing or rehydration process.

Most devitalized heteroplastic grafts behave like foreign bodies. However, masses of nerve fibers use their oriented surface as pathway.

The results indicate that the biophysical and biochemical benefits to regenerative nerve growth inherent in peripheral nerve are essentially preserved in the freezing-drying process. The fitness of devitalized nerve as graft brings the supply problem in nerve grafting nearer its solution. Banks of assorted nerve sizes stored in the dry condition could readily fill a steady demand.

14142 P

Hepatic Injury in Dogs Resulting from a Vitamin B Complex-Free Diet.

VICTOR A. DRILL, C. BOYD SHAFFER, AND JAMES H. LEATHEM. (Introduced by W. W. Swingle.)

From the Section of Physiology, Biological Laboratory, Princeton University, and Department of Zoology, Rutgers University, Princeton and New Brunswick, N.J.

Various authors have studied the effect of administration of the B vitamins in cases of hepatic cirrhosis,[1,2,3] and experimental studies on the relation of the B vitamins, choline, methionine and cystine to hepatic damage have been reported.[4-9] In this paper preliminary studies were made on the effect of a vitamin B complex-free diet on the hepatic function of dogs.

Methods. Seven adult dogs, weighing between 7.9 and 14.3 kg, were used as experimental animals. When first obtained they were placed on a diet of Purina chow, and 2 weeks before the experiment was started they were fed on a modified form[10] of Cowgill's casein diet No. 3,[11] plus a daily yeast supplement. The diet used was free of the B vitamins, the only source of these vitamins in the diet being supplied by the daily yeast supplement. The dogs had access to their food for a 3-hour period each day. The amount of food eaten was measured. Following control measurements of liver function, the yeast was removed from the diet of 5 of the dogs and the liver function, food intake and weight changes studied. The other 2

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[1] Patek, A. J., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 329.

[2] Patek, A. J., and Post, J., *J. Clin. Invest.*, 1941, **20**, 481.

[3] Fleming, R. G., and Snell, A. M., *Am. J. Digest. Dis.*, 1942, **9**, 115.

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[5] Gyorgy, P., and Goldblatt, H., *J. Exp. Med.*, 1939, **70**, 185.

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[11] Cowgill, G. R., *Am. J. Physiol.*, 1928, **85**, 45.

dogs were maintained throughout the experiment on the modified Cowgill diet, plus yeast, as normal controls. The bromsulphalein method of Rosenthal and White,[12] as previously employed with dogs,[10] was used as a test of liver function.

Results. The liver function of the 2 normal dogs varied from 2 to 12% retention of dye at the end of 30 minutes throughout the experiment. We have regarded any dye retention of 15% or greater at the end of 30 minutes as evidence of an abnormal liver function. Four of the 5 dogs used gave evidence of an abnormal liver function when the yeast was removed from the diet. Dog No. 12 showed an abnormal dye retention on the 27th day of the experiment which increased to 60% retention of dye and then declined to normal. A similar result was obtained with dog No. 13 who developed an abnormal liver function on the 42nd day, which increased to 50% retention of dye and then regressed to normal. Dogs Nos. 25 and 30 first gave evidence of an abnormal dye retention on the 28th and 19th day respectively. One dog, No. 27, did not develop an abnormal liver function during the course of the experiment.

At first it seemed strange that the abnormal liver functions of dogs Nos. 12 and 13, once they had developed, would return to normal instead of becoming progressively worse. However, on studying the food intake of the dogs it was observed that the first evidence of abnormal dye retention was obtained just before, or soon after, the dogs developed complete anorexia. It is possible that the continued anorexia would in turn impose less metabolic work upon the liver, which might then have a greater opportunity to excrete the injected dye.

The removal of the yeast from the diet leads to a decrease in appetite which culminates in anorexia. Himwich, Goldfarb and Cowgill[13] obtained anorexia in 4 dogs in an average of 32 days. In this experiment the dogs developed anorexia in an average of 35 days, consuming an average of 1,188 calories per square meter per day before anorexia appeared. Dogs Nos. 13 and 25 developed an abnormal liver function as their appetite was falling, but before complete anorexia ensued. Dogs Nos. 12 and 30 did not develop an abnormal dye retention until after complete anorexia became evident.

On necropsy the gross appearance of the liver was normal in color and size. No mottling or nodularity was present. The liver cut easily and the cut surface appeared normal. The weights of the liver, spleen and kidneys were within the normal range. The heart, however, was hypertrophic, weighing an average of 2 g per kilo of body weight more than normal.

Three different pieces of liver were taken from each dog, fixed in Bouin's solution, and the sections stained with hemotoxylin and eosin. Sections from the liver of dog No. 13 were essentially normal, whereas the livers of the other dogs showed definite pathological changes, varying from a mild to a marked chronic passive congestion. The liver tissue appeared as compact masses with normal nuclei and no evidence of pycnosis. In some sections hyperemia of the sinusoids surrounding the central vein had obliterated the normal hepatic cord appearance. Vacuolization was seen, the cells exhibiting the type swelling and altered cytoplasmic state of cloudy swelling.

Discussion. When the yeast was removed from the diet of the dogs, the increased dye retention did not appear until the food intake had fallen to a low level, or until complete anorexia was present. In this study, therefore, we are dealing with the effect of an acute deficiency of the vitamin B complex, and also with any effect anorexia may have on hepatic function. The results show that as the period of anorexia continued the dye retention returned to normal in 2 dogs. This might tend to show that the lack of food intake as such did not produce the hepatic damage, for if the anorexia was responsible for the abnormal liver function continued anorexia should further increase the liver damage. However, further studies are being made on the effect of anorexia and of a chronic deficiency of the vitamin B complex on liver function.

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A deficiency of yeast or various B vitamins has been reported to produce histological changes in the liver of rats,[4,5,14] rabbits,[15] and guinea pigs.[16] Rhoads and Miller[17] found that there was a lowered capacity of the liver of dogs fed a black-tongue diet to excrete injected bilirubin. The amount of yeast in the diet is also related to the hepatic damage produced by lead arsenate,[18] *p*-dimethyl-amino-azo-benzol[19] and thyroid feeding.[10,20,21] An increased food intake may be a factor in preventing liver damage in such cases.[21,22]

Conclusions. Five dogs fed a vitamin B complex-free diet showed a gradual loss of appetite which culminated in anorexia. Four of these dogs developed an abnormal liver function, as judged by an increased retention of bromsulphalein. The hepatic damage appeared before, or slightly after, the time that anorexia appeared, and as the period of anorexia continued the dye retention returned to normal in 2 dogs. Histologically, the liver of these dogs showed mainly a mild to a marked chronic passive congestion.

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[15] Rich, A. R., and Hamilton, J. D., *Bull. Johns Hopkins Hosp.*, 1940, **66**, 185.

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14143

Ultracentrifugation Studies on the Blood of Normal and Jaundice-diseased Silkworms.

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Through the courtesy of Drs. R. W. Glaser and W. M. Stanley, the author was afforded an opportunity to make a limited number of observations with the ultracentrifuge on the blood of normal and of jaundice-diseased silkworms and on an infectious material isolated by high-speed quantity centrifugation from the fluid of diseased worms. Because it is unlikely that it will be possible to extend this study in the near future, it was thought desirable to describe briefly the results obtained thus far.

A Bauer-Pickels type ultracentrifuge[1,2] equipped with a Svensson-Philpot optical system[3,4] was used in this investigation. The results of sedimentation studies on 4 samples of pooled blood from healthy worms are presented in Table I (Samples I, II, III, and IV). It may be observed that 2 major components were found. As judged by the areas under the schlieren diagrams, the amount of material associated with the slower component was from one-half to one-third of the total.

Three of the samples of normal blood listed in Table I were treated by dilution with an 0.18 M sodium chloride-0.02 M sodium phosphate buffer solution at pH 7, by dialysis against an excess of the buffer, or by both. These treated materials, as well as a prepara-

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[3] Svensson, H., *Kolloid-Z.*, 1939, **87**, 181.

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TABLE I.
Sedimentation Data on Undiluted Silkworm Blood.

Sample	Characterization	Sedimentation constants (Svedberg units)* uncorrected		Temp., °C
I	Fresh normal blood	6.4	10.9	25°
II	” ” ”	6.0	10.8	25°
III	3 yr ” ”	6.6	13.6	27°
IV	” ” ”	6.4	11.8	26°
V	Fresh diseased blood	6.9	13.3	27°
VI	” ” ”	9.4	14.2	—
VII	” ” ”	9.5	15.3	—

* A Svedberg unit, symbolized by S, is a sedimentation rate of 10^{-13} cm per second per unit field.

tion obtained by Glaser and Stanley[5] by centrifuging the fluid of normal silkworms for 2 hours at 60,000 R.P.M. in a Masket[6] type rotor, were examined in the ultracentrifuge. Each was found to contain a fairly homogeneous component and a small amount of more slowly sedimenting material which was too inhomogeneous to give the appearance of a definite boundary. The sedimentation constants corrected to water at 20°C, s^{20}_{w}, of the homogeneous component are listed in Table II. The average of the values listed there is about 16 S. This component is the same as the faster component of the undiluted and undialyzed blood. The values obtained in the untreated whole blood are lower because they were not corrected for the effects of temperature, solvent, and concentration. The inhomogeneous material is evidently composed of the same particles which make up the slower component observed in undiluted and undialyzed silkworm bloods. The addition of buffer and dilution seem to favor the spreading of the boundary of the more slowly moving material. From all of these experiments it can be concluded that the blood from healthy silkworms contains a relatively homogeneous, very stable component with a sedimentation constant, s^{20}_{w}, of about 16 S and a variable amount of very inhomogeneous, more slowly sedimenting material. If the particles with s^{20}_{w} of 16 S are assumed to be spheres of density 1.35, a molecular weight of 325,000 and a particle diameter of 9.1 $m\mu$ may be calculated for them.

TABLE II.
Sedimentation Data on Diluted and Dialyzed Normal Silkworm Blood.

Description of material		s^{20}_{w} Svedberg units
I of Table I	Dialyzed 24 hr	15.2
I ” ” ”	Dialyzed 24 hr, then diluted 1:4 with buffer	16.8
III ” ” ”	Diluted 1:4 with buffer, then dialyzed 24 hr against buffer	16.5
IV ” ” ”	Diluted 1:4 with buffer	16.4
Glaser and Stanley's material isolated from normal worms		15.5

The blood from the diseased worms differed from that of the healthy worms in containing polyhedral bodies[7] and a small amount of fatty material in suspension and in being infectious. The fatty material and the polyhedral bodies were separated from the blood by low-speed centrifugation, and the clarified blood was then examined in the ultracentrifuge. The results obtained with 3 samples of fresh pooled diseased blood clarified as described are presented in Table I (Samples V, VI, and VII). No difference between the clarified blood from diseased worms and the undiluted blood from healthy worms could be observed by this means.

Five different preparations of highly infectious material obtained by Glaser and Stanley[5] by quantity high-speed centrifugation were also examined in the ultracentrifuge. The solvent was 0.10 M borate buffer at pH 7. In every case a single fairly homogeneous component was observed. The sedimentation constants corrected to water at 20° are shown in Table III. The average of the 6 values reported in Table III is about 17 S. This figure is obviously not significantly different from that obtained for the homogeneous constituent of normal fluid. The material it represents must be, of course, the more rapidly sedimenting constituent of the undiluted blood of diseased worms.

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[5] Glaser, R. W., and Stanley, W. M., *J. Exp. Med.*, 1943, **77**, 451.

[6] Masket, A. V., *Rev. Sci. Instruments*, 1941, **12**, 277.

[7] Glaser, R. W., and Lacaillade, C. W., *Am. J. Hyg.*, 1934, **20**, 454.

TABLE III.
Sedimentation Data on Infectious Material from Diseased Silkworms.

Sample	s^{20}_{w} Svedberg units	Sample	s^{20}_{w} Svedberg units
A	17.7	D	19.9
B	17.9	D*	16.8
C	14.4	E	16.4

* After 3 months' storage at 4°C.

The components found in this study to be present in the blood of both healthy and diseased worms are entirely different from the 2 components extracted by Glaser and Wyckoff[8] from the frozen and ground bodies of both healthy and jaundice-diseased silkworms. The blood components have corrected sedimentation constants of 16 or 17 S and about half of that value; the extracts of whole worms showed 2 components with corrected sedimentation constants of 53 and 92 S. The blood constituents are extremely stable, for they are apparently the same in fresh blood and in blood stored in the cold for 3 years; the components extracted from whole worms disintegrate within a few days.

Summary. The blood of normal and of jaundice-diseased silkworms and a highly infectious purified material isolated by Glaser and Stanley[5] from the blood of diseased worms were studied in the ultracentrifuge. No difference between the bloods of healthy and diseased worms was observed by this method. Both contained a homogeneous component with a corrected sedimentation constant of 16 or 17 S and a very inhomogeneous, more slowly sedimenting component. The only component present in an optically detectable amount in the highly infectious purified material was found to have a corrected sedimentation constant of about 17 S.

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14144

Inhibition of Bacterial Growth by Glucose in Media Devoid of Nicotinic Acid.

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In preceding publications[1] it was shown that *Sal. paratyphi* A. and *Sh. dysenteriæ* give appreciable growth in a synthetic or semi-synthetic medium in the absence of nicotinic acid, provided that no sugar or alcohol fermented by these organisms is present. The addition of glucose to such a substrate inhibited growth, while the non-fermentable sucrose (or lactose) had no such unfavorable effect.

The present paper presents data showing that this inhibitive effect obtains with other bacteria requiring nicotinic acid for their utilization of carbohydrates and related substances. In these experiments we tested *B. proteus* XK, three strains of *Sh. dysenteriæ* (Schmitz, Flexner and Hiss), and *Staphylococcus aureus.*

The proteus strain was grown in a medium consisting of the salt solution recommended by Fildes (without glucose or nicotinic acid),

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TABLE I.
Relative Growth of Proteus and Dysentery Organisms in Various Media with and without Nicotinic Acid.

	Proteus XK Synthetic medium		Proteus XK Casein hydrolysate		Flexner X		Schmitz Casein hydrolysate		Hiss	
	Growth density	Plate count (millions)	Growth† density	Plate count	Growth density	Plate count	Growth density	Plate count	Growth density	Plate count
Without nicotinic acid:										
Basic medium	++	180	88	124.6	90	32	87	120	88	116
” and glucose	+	42	97	10.8	99	0.2	100	0.01	99	0.16
” ” lactate	++	140	92	62.6	87	123	88	72	86	200
With nicotinic acid:										
Basic medium	++	590	72	400	78	330	76	370	75	390
” and glucose	+++	620*	68	540	59	1,520	54	1,860	59	1,430
” ” lactate	+++	1,260	66	580	70	690	70	698	67	980
Sterile control	—									

* Many organisms died as a result of the high acidity.
† Photometer readings; sterile tubes = 100.

to which was added 0.3% of an acid hydrolysate of glaxo "ashless" extracted casein: (semi-synthetic medium), or 0.25% asparagine, 0.05% cystine and 0.5% glycine: (synthetic medium).

The dysentery organisms were grown in the following medium: A. Salt solution: NaCl 5.0 g; Na_2HPO_4 2.5 g; K_2HPO_4 0.35 g; H_2O 900 ml; this solution was divided into tubes (4.5 ml per tube) and autoclaved; B. To each tube was added 0.1 ml of a 4% solution of $MgSO_4$ and 0.3 cc of a 10% casein hydrolysate. For staphylococci 0.01% tryptophane was added to this medium.

Parallel growth tests were made in substrates with and without the addition of nicotinic acid (1-2 γ per ml), both in the presence and in absence of glucose (0.1%-0.2%). In the case of staphylococci, which require both nicotinic acid and thiamine, all combinations were tested. The tubes were sown with 1000-4000 washed organisms and incubated in a slanting position for 4 days at 37°C. At the end of that time plate counts and photometric turbidity readings were made. The results are summarized in Tables I and II.

The results show that in the synthetic amino acid medium, in the absence of nicotinic acid, the proteus and dysentery organisms grow 5 to 1000 times more abundantly when no glucose is added than they do in its presence. The staphylococcus grows only sparsely in this medium, but such growth as there is is 100-1000 fold greater in the absence of than in the presence of glucose. It is clear, therefore, that in a nicotinic acid-free medium glucose has an inhibitive effect on growth.

All available evidence indicates that the

TABLE II.
Relative Growth of *Staph. aureus* in Various Media, with and without Nicotinic Acid.

	Basic medium		Basic + 0.2% glucose		Basic + 0.1% lactate		Basic + 0.1% pyruvate	
Medium	Growth density	Count*	Density	Count	Density	Count	Density	Count
Without nicotinic acid:								
+ Thiamine	95	0.95	99	0.006	96	0.13	90	1.10
— Thiamine	98	0.075	100	0.0008	99	0.015	96†	0.095
With nicotinic acid:								
+ Thiamine	60	410.0	49	2,750	57	864.0	55	1,230.0
— Thiamine	79	27.6	65	160	70	97.0	69	103.0

* In millions of colonies per ml.
† The color of the pyruvate influences the photometer readings.

presence of glucose interferes with the oxidation of the amino acids. The quantitative differences in the effect on different organisms, as well as the variable influence of lactic acid, must be presumed to be due to the different capacity of each organism for aerobic oxidation of the hydrocarbons and amino acids. The stimulating effect of nicotinic acid in the amino acid medium without carbohydrates can also be explained on this basis. A more detailed study of the relation of vitamins to the metabolic activity of staphylococci (to be reported elsewhere) indicates that the oxidation of amino acids is similar to that of lactate and pyruvate.

Summary. When nicotinic acid is lacking in a substrate otherwise suitable for growth, the addition of glucose inhibits the growth of organisms capable of fermenting this sugar in the presence of nicotinic acid. The degree of inhibition is 5 to 1000 fold, and depends on the organism and the medium. With Proteus XK the inhibition was 5-10 fold; with dysentery organisms and with staphylococci 100-1000 fold.

14145 P

Effect of Glutamic Acid on the Central Action of the Ammonium Ion.

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In 1936, in a study of the effect of various stimulants and depressants of the central nervous system on the aftercontraction in humans, it was noted that small amounts of ammonium salts (.5 g ammonium), taken by mouth, caused a marked increase in this phenomenon. We have considered the aftercontraction a measure of the excitability of the human cerebral cortex.[1,2,3] It has been repeatedly affirmed in the literature that the concentration of ammonium ion increases in the brain tissue and cerebrospinal fluid in states of increased excitability of humans and animals. The fact that oral and parenteral administration of ammonium salts can produce convulsions in animals is well known.

Since ammonium convulsions so closely simulate spontaneous seizures, it seemed that any substance which would prevent these seizures, might be useful in other types of convulsive activity. The work of Krebs on the metabolism of amino acids in brain tissue slices offered an important clue.[4] Of many substances tested he found that glutamic acid specifically caused a marked reduction in the ammonium ion concentration present in the medium surrounding the tissue slices, due to the action of the enzyme glutaminase which synthesized glutamine from glutamic acid and ammonia. The enzyme system was reversible, was stimulated by the physiological *l* (+) glutamic acid and inhibited by *d* (—) glutamic acid. Under this concept, it should be possible to increase or decrease the ammonium ion concentration of the brain and thus control its convulsive activity. Experiments to test this hypothesis were begun in July, 1942.

In our studies on the role of ammonium ion in the convulsive state, we developed a uniform method of production of seizures in rabbits by the slow intravenous injection of 2½% ammonium chloride solution. One cc per kilo of this solution is administered every 5 minutes until a generalized convulsion results. All 12 of the rabbits (2 kg) used in these experiments had convulsions after 15-25 cc of the solution had been injected. The observations were

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[1] Sapirstein, M. R., Herman, R. C., and Wallace, G. B., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 163.

[2] Sapirstein, M. R., Herman, R. C., and Wallace, G. B., *Am. J. Physiol.*, 1937, **119**, 549.

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[4] Krebs, H. A., *Biochem. J.*, 1935, **29**, 1951.

repeated at weekly intervals to check the thresholds.

We have employed the natural *l* (+) glutamic acid by intravenous injection in an attempt to prevent ammonium convulsions. In a series of preliminary experiments, we have found *l* (+) glutamic acid to give complete protection from the convulsant effects of the ammonium chloride solution. In 8 experiments 50 cc of *l* (+) glutamic acid (5% glutamic acid and 3% sodium bicarbonate) was given intravenously shortly before the injection of ammonium chloride (also dissolved in sodium glutamate solution) and no convulsion resulted in any instance even after 50 cc of the 2½% solution of ammonium chloride was injected. As a control, similar experiments with equimolar amounts of *l* aspartic were performed in the same rabbits. Aspartic acid fails to protect from the convulsant activity of ammonium chloride, indicating the specificity of the glutamic acid reaction.

If *l* (+) glutamic acid is capable of preventing ammonium convulsions, it should be effective in other types of convulsive activity, which are associated with disturbances in ammonium metabolism, perhaps including human epilepsy. We have injected intravenously as much as 500 cc of 5% *l* (+) sodium glutamate into humans within a few hours without any apparent toxic effects. We are now investigating its action in human epileptic seizures.

14146

Blood Histamine in Gastric Cancer and Peptic Ulcer.

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The production of peptic ulcers in experimental animals injected with histamine,[1] and the presence of a gastric secretory depressant in the extracts of achlorhydric cancerous stomachs and juices from such stomachs[2] suggested the investigation of the histamine content of the blood of patients with gastric carcinoma and with peptic ulcer. Histamine determinations in some cases of Hodgkin's disease, leukemia, and polycythemia vera are also reported.

Experimental Procedure. The blood samples were obtained from adult male patients. The diagnosis of gastric carcinoma or of gastric ulcer was established at subsequent operation and by microscopic examination of the tissue. The diagnosis of duodenal ulcer was made clinically on the basis of roentgenographic findings, and the cases of hemorrhagic and atrophic gastritis were studied roentgenographically and gastroscopically. Biopsy of the lymph nodes was made in all cases of Hodgkin's disease.

The histamine content of the whole blood was determined by the method of Barsoum and Gaddum,[3] as modified by Code.[4] The values are expressed as μg of free base per 100 cc of blood.

Results. As given in Table I, the blood histamine in patients with gastric carcinoma and with peptic ulcer was between 1.2 and 8.5 γ per 100 cc. There was no difference between the two groups, and the values did not deviate significantly from the normal range, reported to be between 1.8 and 7.8 γ (average 4.0 γ) in 173 normal individuals

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[1] Hay, L. J., Varco, R. L., Code, C. F., and Wangensteen, O. H., *Surg. Gyn. Obst.*, 1942, **75**, 170.

[2] Brunschwig, A., Schmitz, R. L., and Rasmussen, R., *J. Nat. Cancer Inst.*, 1941, **1**, 481.

[3] Barsoum, G. S., and Gaddum, J. H., *J. Physiol.*, 1935, **85**, 1.

[4] Code, C. F., *J. Physiol.*, 1937, **89**, 257.

TABLE I.
Blood Histamine in Patients with Gastric Carcinoma and with Peptic Ulcer, and Other Conditions.

Case No.	Name	Age	Diagnosis	Leukocytes No./cmm × 100	Neutrophiles* No./cmm	Eosinophiles* No./cmm	Free hydrochloric acid in stomach† degrees	Blood histamine as free base γ/100 cc
1	O.W.	51	Gastric carcinoma	90	6,390	0	0	1.9
2	H.J.	49	” ”	75	5,840	150	0	1.5
3	E.C.K.	42	” ”	74	5,920	75	—	2.8
4	J.A.R.	46	” ”	—	—	—	0	3.9
5	A.G.T.	50	” ”	93	8,000	90	0	4.9
6	S.I.A.	55	” ”	76	4,030	0	42	4.4
7	W.F.B.	46	” ”	74	5,330	130	0	6.7
8	W.P.G.	42	” ”	56	3,280	55	0	6.0
9	W.W.	63	” ”	51	2,040	0	0	1.2
10	R.B.L.	57	” ”	69	3,190	0	0	2.1
11	J.H.S.	66	Gastric ulcer	107	8,990	0	80	2.6
12	J.D.	43	” ”	75	4,420	225	25	5.0
13	W.McK.	51	” ”	122	9,800	120	35	5.3
14	W.K.	49	” ”	93	6,550	185	15	4.4
15	H.S.	48	” ”	48	2,670	100	30	2.9
16	E.S.P.	46	” ”	120	8,430	0	25	2.6
17	H.W.	54	Duodenal ulcer	96	6,180	290	76	6.0
18	J.P.W.	62	” ”	—	—	—	40	8.5
19	R.C.D.	46	” ”	114	9,570	0	0	5.4
20	C.J.L.	36	” ”	62	2,910	125	40	5.5
21	C.J.O'L.	44	” ”	63	5,230	0	25	1.4
22	C.J.	49	Hemorrhagic gastritis	54	3,350	110	0	5.0
23	G.C.	52	Atrophic gastritis	113	7,910	115	0	5.0
24	J.H.D.	53	Hodgkin's disease	26	2,110	35	—	1.9
25	J.S.G.	27	” ”	117	8,460	175	—	3.9
26	P.W.	66	” ”	37	2,000	220	—	6.0
27	H.S.McW.	46	” ”	116	9,860	0	30	1.4
28	C.H.	20	” ”	68	5,460	410	—	8.3
29	E.O.L.	19	” ”	188	16,590	0	—	1.2
30	I.G.T.	78	Mycosis fungoides	84	6,720	505	—	2.3
31	W.D.B.	46	Polycythemia vera	196	16,860	1,175	—	12.6
32	E.E.	44	Chronic myeloid leukemia	2,961	254,500‡	11,800§	30	575.0
33	J.E.T.	35	Acute myeloid leukemia	850	79,050‡	0	—	3.2
34	H.E.A.	62	Chronic lymphatic leukemia	965	3,860	0	35	4.6

* Calculated from differential counts of 100-200 cells.
† 20-30 minutes after 1.0 mg of histamine subcutaneously; cases 17 and 21, fasting.
‡ Including premyelocytes and myeloblasts.
§ Including eosinophilic premyelocytes.

studied by Haworth and Macdonald,[5] Rose,[6] and Randolph and Rackemann.[7] There was no correlation between the histamine of the blood and the presence or absence of free hydrochloric acid in the stomach.

No constant alteration in blood histamine was encountered in patients with Hodgkin's disease, chronic lymphatic leukemia, and mycosis fungoides. The blood histamine was definitely elevated in the patient with polycythemia vera.

In the patient with chronic myeloid leukemia the blood histamine was one hundred times above normal. The white blood cell count was 296,150, with 46% neutrophiles, 23% premyelocytes, 22% myeloblasts, 2% eosinophiles and 3% eosinophilic premyelocytes. Extremely high blood histamine levels in chronic myeloid leukemia have been reported by Code and Macdonald[8] and others.

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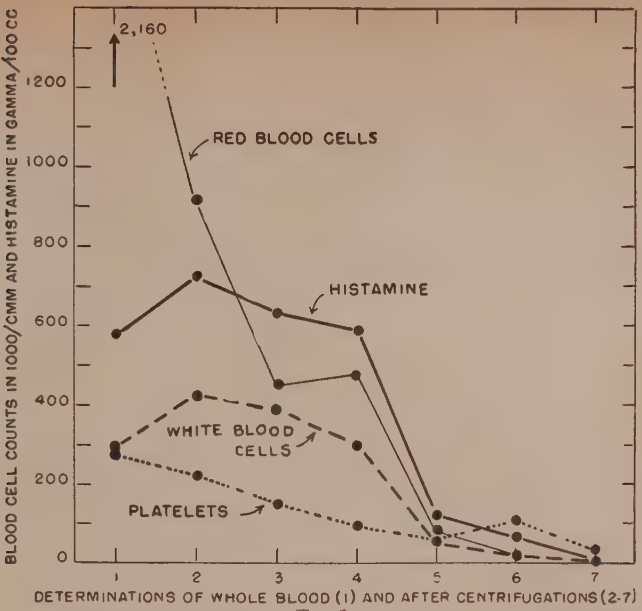

FIG. 1.

Relation of histamine to the number of blood cells in whole blood (1) and in washed blood cells after serial centrifugations (2-7), in a patient with chronic myelogenous leukemia.

The patient with acute myeloid leukemia died 2 months after the onset of illness. He had been treated with Fowler's solution. The white blood cell count was 85,000, of which 44% were neutrophiles, 27% premyelocytes, and 22% myeloblasts; no eosinophiles were seen among 400 cells. The blood histamine in this patient was normal. It is possible that the finding is attributable to the treatment with arsenic or the absence of eosinophiles. It would be of interest, however, to determine the blood histamine in additional cases of acute myeloid leukemia as compared with chronic myeloid leukemia.

Code and Macdonald[8] and others have established that the blood histamine in man is carried chiefly by the granulocytes. This is well shown by separation experiments carried out on the blood of the patient with chronic myeloid leukemia, in whom the high histamine facilitated the determinations in various fractions of the blood separated by centrifugation. Fig. 1 shows that the blood histamine closely paralleled the number of white blood cells. The reduction of the red cells from 2,160,000 to 425,000 and the removal of most of the plasma did not alter the histamine level. The blood histamine fell sharply between the fourth and sixth centrifugations, despite the maintenance of the platelets at 100,000 per cmm.

The normal blood histamine in chronic lymphatic leukemia and the low histamine in lymph nodes[8] indicate that the lymphocytes do not contain appreciable amounts of histamine. Randolph and Rackemann[7] concluded that there was no constant relationship between the number of eosinophiles and blood histamine, although Code and Macdonald[8] reported raised values in patients with eosinophilia. In one patient with eosinophilia (34% of 10,500 white blood cells) studied here, the blood histamine was 7.9 γ.* It is interesting, however, that the eosinophiles were increased in the 2 patients with elevated blood histamine (31 and 32), although the analysis of the remainder of the data shows little, if any, correlation between blood histamine and the number of eosinophiles. The data also show no constant relationship between the number of neutrophiles and blood histamine. As further evidence may be cited a patient whose blood histamine was 8.8 γ with a white blood cell count of 5,480; after an injection of typhoid vaccine, resulting in an increase of the white cells to 12,650, the blood histamine dropped to 3.7 γ.

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* In this and in the subsequent case mentioned but not included in the table, the blood was not extracted with absolute alcohol saturated with sodium chloride. The values are higher than after alcohol extraction, but wide fluctuations in blood histamine are adequately shown by either method.[9]

[9] Zon, L., Ceder, E. T., and Crigler, C. W., *Pub. Health Rep.*, 1939, **64**, 1978.

Summary. Blood histamine is within normal limits in patients with gastric carcinoma and with peptic ulcer. There is no relationship between blood histamine and the presence or absence of free hydrochloric acid in the stomach.

Blood histamine in man is carried chiefly by granulocytes, but is not directly related to the number of granulocytes in the blood.

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Bacterial Synthesis of *p*-Aminobenzoic Acid.

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Fildes[1] and Woods[2] have postulated that the sulfonamide drugs prevent bacterial growth by interfering with the utilization of an "essential metabolite,"* *p*-aminobenzoic acid (PAB), by virtue of their similarity in structure to this compound. At the time this theory was proposed it was assumed that PAB was an essential metabolite for bacteria, although for technical reasons, it had not yet been isolated from them, nor had it been proved to be a growth factor in the absence of a bacterium which could not synthesize it. Recent investigations have done much to substantiate Fildes' conception of the role of PAB in sulfonamide bacteriostasis.[3,4] PAB has been found to be a growth essential for *Clostridium acetobutylicum*,[5] *Acetobacter suboxydans*[6] and evidence now indicates that it may be a growth factor for certain Lactobacilli[7,8] and Corynebacteria[9] as well.

If PAB is truly an essential metabolite, the fact that it is a growth factor for a few organisms should be supplemented by evidence that it is important in the nutrition of bacteria generally, namely proof of its synthesis by organisms able to grow in PAB-free culture media. Analogy to other growth factors indicates that this is a situation which might well exist. It has been repeatedly observed that while a few organisms may require the presence of growth factors, others (the majority) usually synthesize these vitamins in appreciable amounts.[10-13]

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[1] Fildes, P., *Lancet*, 1940, **1**, 955.

[2] Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

* According to Fildes[1] an essential metabolite is a substance which takes an essential part in a chain of syntheses necessary for bacterial growth. A "growth factor" which must be supplied in the nutrients is an essential metabolite which the cell cannot synthesize, *e.g.*, nicotinic acid is an essential metabolite for all bacteria but a growth factor for only a few.

[3] Green, H. N., and Bielschowsky, F., *Brit. J. Exp. Path.*, 1942, **23**, 1.

[4] McIlwain, H., *Brit. J. Exp. Path.*, 1942, **23**, 265.

[5] Rubbo, S. D., Maxwell, M., Fairbridge, R. A., and Gillespie, J. M., *Austral. J. Exp. Biol. and Med.*, 1941, **19**, 185.

[6] Lampen, J. O., Underkofler, L. A., and Peterson, W. H., *J. Biol. Chem.*, 1942, **146**, 277.

[7] Isbell, H., *J. Biol. Chem.*, 1942, **144**, 567.

[8] Lewis, J. C., *J. Biol. Chem.*, 1942, **146**, 441.

[9] Chattaway, F. W., Happold, F. C., Lythgoe, B., Sandford, M., and Todd, A. R., *Biochem. J.*, 1942, **36**, vi.

The importance of PAB in determining the susceptibility or resistance of bacteria to sulfonamide bacteriostasis, together with the recent availability of an adequate microbiological method for PAB assay[14] led us to investigate its production by bacteria. This report is a summary of data indicating that PAB is, in fact, synthesized by bacteria of many diverse species.

Experimental. The culture medium employed for growth of most of the organisms investigated was synthetic in composition and free of PAB (by test with *A. suboxydans*). The composition of this medium is given in Table I.

TABLE I.
p-Aminobenzoic Acid-Free Culture Medium.

Glucose	1.0	g
Casein hydrolysate, purified	0.5	”
Tryptophane	10	mg
Cystine	10	”
K_2HPO_4	50	”
KH_2PO_4	50	”
$MgSO_4 \cdot 7H_2O$	20	”
NaCl	1	”
$FeSO_4 \cdot 7H_2O$	1	”
$MnSO_4 \cdot 2H_2O$	1	”
Adenine sulfate	500	µg
Thiamine	1	”
Folic acid	0.5	”
Biotin	0.5	”
Riboflavin	20	”
Calcium pantothenate	20	”
Nicotinic acid	20	”
Pyridoxine	40	”
Distilled water to	100	cc
pH adjusted to 7.5		

As will be indicated below, other culture media were required in special instances; however, most of the tests were made with this medium.

Since the streptococci and pneumococci grew poorly, or failed to grow, in the above medium (Table I), it was necessary to use a special procedure for their cultivation. A medium which was satisfactory in supporting their growth and at the same time free of PAB was prepared by treating a 2% solution of Bacto Tryptose with Darco G-60 charcoal (2% by weight of Tryptose solution) at pH 5.0, bringing to a boil and filtering while hot.[15] The charcoal treated tryptose solution was supplemented as follows:

TTV Medium (Treated Tryptose and Vitamins).

2% Tryptose solution, charcoal treated	100	cc
Glucose	.1	g
Sodium thioglycollate	.01	”
Tryptophane	.005	”
Thiamine	10	µg
Riboflavin	25	”
Calcium pantothenate	25	”
Nicotinic acid	25	”
Pyridoxine	40	”
Biotin	.1	”
Inorganic salts (same as in Table I)		
pH 7.6		

This medium supported growth of streptococci and pneumococci equivalent to that obtainable with meat infusion broth. The anaerobes were cultured in a simplified medium devised by Dr. Sarah E. Stewart of the National Institute of Health. The medium contained purified casein hydrolysate, crystalline vitamins, tryptophane, cystine and pyruvic acid. The pH was adjusted to 7.6.

The organisms listed in Table II were grown in the various culture media indicated and transferred a number of times to minimize the possibility of carrying over any PAB from the original media. The inoculum size was restricted to a minimum. Incubation was at 37°C for 24 hours, except where otherwise indicated. Although it was not possible to obtain optimum growth of all the organisms tested, growth was generally good.

In preparation for PAB assay, half of each culture was filtered through a Seitz filter while the other half was adjusted to pH 5.0 and hydrolyzed by autoclaving at 15 lb pressure for one-half hour. Both filtrate and hydrolysate were adjusted to pH 6.0 and their PAB content determined by microbiological assay. The PAB values for cells given in Table II represents the difference obtained by subtracting the PAB content of the medium from

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[10] Snell, E. E., and Strong, F. M., *Enzymologia*, 1939, **6**, 186.

[11] Landy, M., and Dicken, D. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 499.

[12] Burkholder, P. R., and McVeigh, I., *Proc. Nat. Acad. Sci.*, 1942, **28**, 285.

[13] Thompson, R. C., *Univ. Texas Publ.*, 1942, **4237**, 87.

[14] Landy, M., and Dicken, D. M., *J. Biol. Chem.*, 1942, **146**, 109.

[15] MacLeod, C. M., and Mirick, G. S., *J. Bact.*, 1942, **44**, 277.

TABLE II.
Bacterial Synthesis of *p*-Aminobenzoic Acid.

Organism	Medium	Incubation		*p*-Aminobenzoic acid found (γ per cc culture)		
		°C	Hr	In cells	In medium	Total
Salmonella paratyphi	Synthetic (Table I)	37°	24	.001	.019	.020
Salmonella schottmuelleri	”	”	”	.021	.029	.050
Shigella dysenteriæ	”	”	”	0	.018	.018
Eberthella typhosa	”	”	”	0	.018	.018
Escherichia coli	”	”	”	.038	.007	.045
Brucella abortus	”	”	72	0	.013	.013
Proteus vulgaris	”	”	24	.001	.019	.020
Pseudomonas aeruginosa	”	”	”	.010	.050	.060
Aerobacter aerogenes	”	”	”	.017	.028	.045
Bacillus subtilis	”	”	”	0	.019	.019
Bacillus megatherium	”	30°	24	0	.007	.007
Serratia marcescens	”	”	”	.007	.007	.014
Bacillus vulgatus	”	”	”	.041	.030	.071
Klebsiella pneumoniæ	”	37°	”	.010	.032	.042
Alkaligenes fecalis	”	”	48	.001	.005	.006
Corynebacterium diphtheriæ	”	”	24	.070	.171	.241
Staphylococcus albus	”	”	”	.014	.047	.061
Staphylococcus aureus	”	”	”	.004	.063	.067
Vibrio choleræ	”	”	”	—	.022	—
Shigella paradysenteriæ	”	”	”	.006	.011	.017
Mycobacterium stercoris	Long and Seibert	”	96	.003	.019	.022
Mycobacterium smegma	”	”	”	.007	.052	.059
Mycobacterium tuberculosis	”	”	720	—	.200	—
*Saccharomyces cerevisiæ**	Snell, Eakin and Williams	”	16	—	—	.180
Streptococcus hemolyticus	TTV	”	24	.008	.004	.012
Streptococcus salivarius	”	”	”	.005	.005	.010
Streptococcus scarlatinæ	”	”	”	.020	.030	.050
Diplococcus pneumoniæ I	”	”	”	.001	.004	.005
Diplococcus pneumoniæ II	”	”	”	.001	.003	.004
Diplococcus pneumoniæ III	”	”	”	.002	.003	.005
Lactobacillus casei	Landy and Dicken	”	72	—	.016	.016
Lactobacillus arabinosus†	Snell and Wright	30°	”	0	0	0
Clostridium botulinum	Stewart	37°	144	—	—	.004
Clostridium sporogenes	”	”	48	—	—	.003
Clostridium tetani	”	”	”	—	—	.008

* As determined by reversal of sulfonamide inhibition of yeast growth, Landy and Dicken[16] estimated that *Saccharomyces cerevisiæ* No. 139 cultured in the medium of Snell, Eakin and Williams produced "a neutralizing substance equivalent to approximately 1.5 μg of PAB (10 cc of culture)." This is in good agreement with the direct PAB assay which found 0.18 μg PAB per cc of culture.

† Approximately one-half maximum growth (as measured by acid production) was obtained in the Snell and Wright medium prepared with highly purified casein hydrolysate. By microbiological assay this medium contained less than .0001 μg of PAB per cc. Since *L. arabinosus* has been shown to require PAB for growth, sufficient PAB must still have been present in the medium to support the growth obtained. It is significant, however, that the organism did not synthesize any detectable PAB.

that of the hydrolyzed whole culture.

Discussion. The organisms studied included the greatest variety of bacterial types which could be adequately cultured in PAB-free media. It is important to emphasize that the values for PAB synthesis thus obtained apply only to our test conditions. Since it is known that the culture media were not equally satisfactory for the growth of the various organisms studied, it is not justifiable to compare PAB synthesis by different bacterial species. The effect of various nutrients on production of PAB by bacteria is quite unknown and it is possible that under other cultural conditions different assay values might be obtained.

Examination of Table II reveals that most bacteria synthesize PAB in readily measurable amounts. It will be observed that in most instances the bulk of the PAB produced is found in the culture medium. Few of the species retain any great amount of the PAB in their cells. The quantity of PAB made by bacteria appears to be variable since it was observed that strains of a given species cultured under apparently identical conditions did not necessarily yield the same amount of PAB. These results are, therefore, only indicative of the ability of bacteria to elaborate PAB and are not intended to convey any quantitative comparison of various organisms.

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[16] Landy, M., and Dicken, D. M., *Nature*, 1942, **149**, 244.

The present significance of PAB synthesis by microörganisms is twofold. (1) It is additional evidence for a general biological phenomenon observed in the case of other growth factors, namely, synthesis by bacteria of vitamins when grown in a medium free of such compounds. (2) It has been suggested that the "sensitivity" of a bacterium to sulfonamides would depend, at least in part, upon whether it could synthesize PAB readily or not.[†] An organism with little synthetic ability would be more sensitive to sulfonamides than one with greater synthetic powers.[2,3] The finding that bacteria synthesize PAB in variable degree appears to make this suggestion a definite possibility. In any case it is likely that the PAB synthesis by a given organism would play some part in the degree of its resistance to sulfonamide action.

That PAB is generally important in bacterial metabolism is indicated by the fact that the organisms tested, of many diverse species, all[‡] synthesized PAB. It is not likely that so many unrelated types of organisms would all make this compound unless it is an "essential metabolite" as defined by Fildes. It is likely that PAB is synthesized by many other organisms as well, organisms for which we have been unable to devise suitable PAB-free culture media for test.

Summary. Representative cultures of many bacterial genera were grown in culture media of known composition free of *p*-aminobenzoic acid. Culture filtrates and hydrolized whole cultures were assayed for *p*-aminobenzoic acid content employing the *Acetobacter suboxydans* microbiological method. All organisms successfully cultured in the *p*-aminobenzoic acid free media elaborated *p*-aminobenzoic acid in readily measurable amounts, indicating that *p*-aminobenzoic acid is truly an "essential metabolite" synthesized by many bacteria.

[†] This association would appear to have been established in at least one instance. We have observed[17] that sulfonamide resistant *Staphylococcus aureus* cultures synthesize far more (approximately 70 times as much) PAB than their parent (susceptible) strains. On the other hand, we could not detect any significant difference in PAB production by sulfonamide susceptible and resistant strains of *Escherichia coli, Shigella dysenteriæ, Vibrio choleræ* and *Diplococcus pneumoniæ*, Types I, II and III.[18]

[17] Landy, M., Larkum, N. W., Oswald, E. J., and Streightoff, F., *Science*, 1943, **97**, 265.

[18] Landy, M., Larkum, N. W., Oswald, E. J., and Streightoff, F., unpublished data.

[‡] See footnote † to Table II.

14148

Influence of Pregneninolone and Pregnenolone on Spermatogenesis in Hypophysectomized Adult Rats.*

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An androgenic action is ascribed to the steroid pregneninolone on the basis of its ability to increase the size of the capon's comb,[1] to stimulate the preputial glands and clitoris of the female rat,[2] to increase the weight of the seminal vesicles, prostates and coagulating glands of both the prepubertal and adult castrate rat[3] and to cause precocious assumption of male characters in Lebistes.[4] The maintenance of spermatogenesis with androgens following hypophysectomy is well established[5-7] and it was deemed of interest to test pregneninolone in hypophysectomized male rats to ascertain the action on the reproductive system.

Another steroid, pregnenolone, though shown by Selye and Albert[8] to be practically inactive as an androgen, in both the immature and adult castrate rat, was included in this investigation because it was felt that this substance might have a gonadotropic effect. Previous investigations have demonstrated that pregnenolone will inhibit testicular atrophy induced by both testosterone and estradiol[9,10] and recently Selye[11] indicated but presented no data that this substance prevented testicular atrophy following hypophysectomy.

Adult male rats ranging from 120 to 200 days in age were hypophysectomized and treated for a 20-day period starting the day of operation. All rats were autopsied on the 21st day, and organ weights of fresh tissues were taken. Seminal vesicle weight included the coagulating gland and any contained fluid. All pituitary capsules were serially sectioned, stained with Mallory's connective tissue stain and examined microscopically. Data from only completely hypophysectomized rats are presented.

Pregneninolone (Δ^4-Pregnene-in-20-ol-17-one-3) was initially tested by implanting subcutaneously, on the day of operation, 2 pellets of crystalline hormone weighing 20 mg each. After 20 days the recovered pellets weighed 18.0 to 18.5 mg indicating only slight absorption. The testes from the 3 rats were markedly atrophic and spermatozoa were absent.

In view of the small amount of hormone absorbed from the pellets, the steroid was then tested by the daily subcutaneous injection of 10 mg suspended in 0.2 cc of peanut oil. This was accomplished with some difficulty and usually involved the addition of another 0.2 cc of oil in an attempt to obtain all of the hormone from the ampul. After a 20-day injection period the testes had regressed virtually to control weight and the seminiferous tubules were devoid of spermatozoa. It should be noted that seminal vesicle weights are greater than those of the operated control rats, but this can only be suggestive of stimulation because the number of rats tested was small (Table I).

Pregnenolone (Δ^5-Pregnene-ol-3-one-20) was also administered to hypophysectomized rats as a daily subcutaneous injection of 10 mg suspended in 0.2 cc of peanut oil. Testis weight was well maintained during the 20-day test period, being more nearly comparable to that of the normal rat than of the hypophysectomized control rat (Table I). Seminiferous tubules were large and spermatozoa were numerous. However, desquamated cells were observed in the lumina of numerous tubules suggesting that maintenance of spermatogenesis was not completely normal. Interstitial tissue was atrophic.

Pregnenolone was also tested by injecting 10 mg subcutaneously every other day. The administration of this 100 mg total dosage did not maintain testis weight quite as well as the 200 mg dosage but spermatogenesis was maintained, spermatozoa being numerous.

In view of the marked effect of pregnenolone on the testis it was interesting to note that seminal vesicle weight was not influenced by this steroid (Table I). Furthermore, the typical loss in adrenal and thyroid weight resulting after hypophysectomy was not influenced by the injection of either steroid.

The disappearance of the adrenal X-zone

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* Supported by a grant from the Sigma Xi Alumni Research Fund made to J. H. Leathem.

[1] Emmens, C. W., and Parkes, A. S., *Nature*, 1939, **143**, 1064.

[2] Salmon, U. F., and Salmon, A. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 709.

[3] Selye, H., and Albert, S., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 361.

[4] Eversole, W. J., *Endocrinology*, 1941, **28**, 603.

[5] Walsh, E. L., Cuyler, W. K., and McCullagh, D. R., *Am. J. Physiol.*, 1934, **107**, 508.

[6] Nelson, W. O., and Gallagher, T. F., *Science*, 1936, **84**, 230.

[7] Cutuly, E., McCullagh, D. R., and Cutuly, E. C., *Am. J. Physiol.*, 1937, **119**, 121.

[8] Selye, H., and Albert, S., *J. Pharm. Exp. Therap.*, 1942, **76**, 137.

[9] Selye, H., and Albert, S., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 227.

[10] Albert, S., and Selye, H., *J. Pharm. Exp. Therap.*, 1942, **75**, 308.

[11] Selye, H., *Endocrinology*, 1942, **30**, 437.

TABLE I.
Influence of Steroids on Hypophysectomized Male Rats.

Treatment total Dosage, mg	No. of rats	Body st., g	Weight end, g	Avg organ wt (mg)			
				Testis	Sem. ves.	Thyroid	Adr.
			Pregneninolone.				
40	3	305	232	887	203	14.8	9.6
200	3	276	207	901	249	13.0	5.2
			Pregnenolone.				
100	3	274	222	1645	145	14.3	6.8
200	6	303	242	1944	151	16.0	6.8
Operated controls	6	281	200	708	133	13.7	7.2
Normal controls	6	240	277	2723	813	25.9	28.2

of the mouse can readily be produced with testosterone propionate. Indeed, Starkey and Schmidt[12] have shown that a single injection of 0.4 mg of this hormone will cause the disappearance of the X-zone at the 6th day. To further test pregnenolone for androgenic activity, eleven 25-day-old female mice were spayed and tested 7 days later. Three mice received a single subcutaneous injection of 10 mg of pregnenolone, 4 mice received 5 mg and 4 mice served as controls (caged separately). All mice were autopsied 10 days later and the weights of the uteri, adrenals and kidneys were nearly identical with those of the control mice. Furthermore, the adrenal X-zone was present and large in all mice indicating the absence of androgenic activity under the experimental conditions cited.

Summary. Pregnenolone is capable of maintaining spermatogenesis for at least 20 days following hypophysectomy. However, this steroid does not influence the seminal vesicles, since the loss in seminal vesicle weight simulates that observed in the hypophysectomized control. Pregnenolone therefore appears to be gametogenic in action.

A single injection (10 mg) of pregnenolone did not influence uterine weight or the adrenal X-zone of the spayed mouse.

Pregneninolone, which has been shown to exhibit androgenic activity, failed to maintain spermatogenesis in the hypophysectomized rat.

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[12] Starkey, W. F., and Schmidt, E. C. H., *Endocrinology*, 1938, **23**, 339.

14149 P

Vitamin A Absorption in Infantile Eczema.

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It is commonly observed that while most infants with eczema are overnourished, a few intractable cases appear to be underdeveloped and underweight. In most instances, this malnutrition is probably due to prolonged secondary infections, but occasionally this lack of growth and development seems to be primary and perhaps associated, according to some authors, with a disturbance in the physiology of gastro-intestinal tract.

During a period of several months, 4 intractable cases were seen in this hospital. The course of each was characterized by (a) retarded development, (b) malnutrition, (c) severe and more or less generalized eczema (d) pronounced lymphadenopathy (e) marked eosinophilia (between 25% and 35%), (f) protracted respiratory infections with frequent episodes of broncho-pneumonia (low grade and present for several months in 2 cases), and finally (g) lack of response to topical and dietetic treatment.

TABLE I.
Blood Vitamin A Absorption Curves in Infants with (Cases 5 to 8) and without (Cases 1 to 4) Eczema.

Case No.	Age (mo)	Sex	Diagnosis	Vit. A blood levels (blue units) in hrs				
				0	3	6	9	12
1	54	M	Neurodermatitis of popliteal spaces	5	250	200	100	25
2	3	F	Cleft palate	5	100	300	100	
3	18	M	Acute mastoiditis	25	100	30	30	
4	1	F	Impetigo contagiosa	5	75	50	75	5
Mean				10	131	145	76	15
5	14	M	Infantile eczema	13	25	50	40	10
6	11	M	Infantile eczema	11	66	41	10	5
7	7	F	Infantile eczema	8	12	19	16	7
8	5	M	Infantile eczema	5	50	25	12.5	5
Mean				9	38	34	20	7

It appeared possible that the respiratory infections and malnutrition could be accounted for on the basis of vitamin A deficiency resulting from a defect in intestinal absorption (frequently seen in patients with cystic fibrosis of the pancreas). Vitamin A absorption tests, therefore, were carried out in these 4 infants and in 4 control cases.

Methods. The procedures used were those described by Clausen and McCoord[1] and by May and McCreary.[2] Vitamin A blood levels were determined before and at stated intervals after the ingestion of oleum percomorphum. The dose of fish oil concentrate varied according to body weight (0.1 cc of oleum percomorphum representing 6,000 units of vitamin A per pound of body weight). The vitamin A content of the blood was estimated by a modification of the Price-Carr reaction as described by Clausen and McCoord.[1]

Results. Table I shows the results obtained. The vitamin A blood levels in the infants with eczema (cases 5 to 8) were relatively flat as compared to those obtained in the control cases (cases 1 to 4). The mean values for the two groups are indicated in the table and show the striking differences observed.

Conclusion. In 4 infants with intractable eczema associated with retarded development and malnutrition, the absorption of vitamin A from the intestinal tract was found to be impaired.

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[1] Clausen, S. W., and McCoord, A. B., *J. Pediatrics,* 1938, **13**, 635.

[2] May, C. D., and McCreary, J. F., *J. Pediatrics,* 1941, **18**, 200.

14150

Lipids of Muscle and Brain in Rats Deprived of Tocopherol.

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The changes in chemical composition of tissues associated with vitamin E deficiency include alterations in the amounts of the lipid components. In rabbits with muscular dystrophy Morgulis, Wilder, Spencer, and Eppstein[1] found pronounced increases in the total lipids and especially in cholesterol, and the increasing proportion of cholesterol esters ap-

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[1] Morgulis, S., Wilder, V. M., Spencer, H. C., and Eppstein, S. H., *J. Biol. Chem.,* 1938, **124**, 755.

TABLE I.
Average Amounts of Lipid in Rat Muscle and Brain as Per Cent of Dry Substance.

Numbers and kinds of animal	Cholesterol				Cholesterol in total lipids,
	Free	Total	% free	Total lipids	%
Muscle					
Normal (12)	.29 (.23-.37)	.34 (.25-.42)	84	7.7 (2.9-15.4)	4.5
Control (7)	.22 (.20-.25)	.22 (.20-.25)	100	6.9 (5.7-8.6)	3.4
E-deficient:					
Moderate (7)	.35 (.26-.56)	.38 (.27-.57)	87	9.1 (7.6-11.6)	4.2
Severe (6)	.56 (.46-.79)	.55 (.48-.74)	100+	10.9 (8.4-15.3)	5.2
Brain					
Normal (15)	4.00 (1.83-7.96)	5.83 (4.54-8.10)	61	36.9 (27.6-47.2)	15.0
Control (7)	4.32 (2.86-6.99)	6.66 (5.43-8.33)	65	37.0 (29.9-47.8)	18.3
E-deficient:					
Moderate (8)	7.47 (6.14-8.18)	7.96 (6.88-10.20)	95	53.3 (44.4-61.1)	15.0
Severe (6)	7.01 (6.16-7.67)	7.51 (7.04-8.90)	93	26.3 (12.4-39.5)	35.1

peared to be a specific characteristic of dystrophic muscle. In nursling rats the muscular manifestations of vitamin E deficiency are equally acute but in half-grown and older rats they appear more gradually. In such animals creatine and chloride in muscle are altered as in the typical acute dystrophy of rabbits, and it was therefore of interest to know whether the lipid changes in muscle and brain of rats were similar to those that take place in rabbits.

The vitamin E deficient rats were maintained from weaning on the following diet: casein 18, sucrose 45.5, lard 22, salt mixture 2.5, yeast 10, and cod liver oil 2%. Control animals were fed the same diet, with wheat germ oil substituted for 5% of the lard, and normal animals from a stock diet of dog chow were also included. All of the animals were full-grown or nearly so; those that had been on the diet longest showed the most pronounced external signs of the deficiency.

The animals were killed with sodium amytal. The entire brain was mixed thoroughly. The right gastrocnemius muscle was minced in a tissue mincer;[2] minced muscle gave significantly higher cholesterol values than identical samples diced with scissors, probably due to more complete extraction. Part or all of the ground tissue was then transferred to weighed 10 ml glass-stoppered cylinders for drying and subsequent extraction, according to the method of Sturges and Knudson.[3] Free and total cholesterol were determined in duplicate on aliquot portions of the extract by the micromethod of Schoenheimer and Sperry.[4]* For precipitation of cholesterol, aqueous digitonin as here used was found to be more satisfactory than the alcoholic solution.[3,5] Occasionally, with either solution, there was incomplete precipitation or precipitation of interfering substances as evidenced by turbidity; results of analyses in which this occurred are omitted. Total lipids were determined by drying an aliquot of the extract at 90° to constant weight.

As seen in Table I, total and free cholesterol in muscle were significantly increased in dystrophy but the proportions were not greatly altered. Total lipids gradually increased as dystrophy progressed but not quite as rapidly as cholesterol, such that in severe dystrophy the proportion of cholesterol in the total lipids was slightly but perhaps not significantly increased. In brain tissue of dystrophic animals cholesterol, both free and total, markedly increased, especially the free—such that the proportion of cholesterol esters was insignificant. Total lipids increased in moderate dystrophy; they were subnormal in amount in severe dystrophy and the significance of the high proportion of cholesterol in total lipids is not understood. These results are in general similar to those obtained in rabbits,[1] with 2 or 3 exceptions. One of these is the marked decline in the total lipids in the brain of severely dystrophic rats just mentioned. The course of the disease in rats and in rabbits is dissimilar and the chronic condition in which rats, for months, miserably drag themselves around to secure food and drink, is very different from the complete prostration of rabbits. The brain tissue of normal and control rabbits contains practically no esterified cholesterol, whereas in normal and control rats, about a third of the total cholesterol was esterified. Finally, the differences in muscle cholesterol as between normal and control animals may be related to the high fat diet of the latter. Furthermore, some dog biscuits are not a rich source of vitamin E[6] and the control animals were more generously supplied with it than the normal animals. The significance of these lipid changes will not be understood until the role of vitamin E in oxidative processes[7] is clarified.

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[2] Seevers, M. H., and Shideman, F. E., *Science*, 1941, **94**, 351.

[3] Sturges, S., and Knudson, A., *J. Biol. Chem.*, 1938, **126**, 543.

[4] Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, 1934, **106**, 745; Sperry, W. M., *Am. J. Clin. Path.*, Tech. Suppl., **1938, 2, 91.**

* We are indebted to Dr. Morgulis for the loan of the Wratten filter required.

[5] Kelsey, F. E., *J. Biol. Chem.*, 1939, **127**, 15.

• *Summary.* In rats on a vitamin E-deficient diet the cholesterol content of muscle was significantly increased, the total lipid less markedly. In brain tissue the cholesterol content was markedly increased, especially that of the free cholesterol, such that cholesterol esters represented about 5% of the total; in control animals this figure was 35%. These changes generally parallel those reported for rabbit tissue but are not as striking, perhaps because in grown rats dystrophy develops slowly. An understanding of these changes must await further knowledge of the functional activity of vitamin E.

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[6] Mason, K. E., *J. Nutrition*, 1942, **23**, 71.

[7] Houchin, O. B., *J. Biol. Chem.*, 1942, **146**, 313; Kaunitz, H., and Pappenheimer, A. W., *Am. J. Physiol.*, 1943, **138**, 328.

14151

Occurrence of Premature Ovulation in the Domestic Fowl Following Administration of Progesterone.*

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During the course of tests undertaken to determine the possible value of steroids in controlling the release of pituitary gonadotrophins in fowl, it was unexpectedly found that progesterone, injected under appropriate conditions, regularly caused the premature ovulation of ovarian follicles in laying hens.

Progesterone is known to induce ovulation in amphibia,[1,2,3] but we are not aware that a similar result has been obtained previously either in birds or in normal mammals. Pearl and Surface[4] concluded that the administra-

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* This investigation was carried out under grant of funds provided by the Bankhead-Jones Act.

[1] Shapiro, H. A., *Chem. Indus.*, 1936, **55**, 1031.

[2] Zwarenstein, H., *Nature*, 1937, **139**, 112.

[3] Langan, W. B., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 59.

[4] Pearl, R., and Surface, F. M., *J. Biol. Chem.*, 1914, **19**, 263.

tion of a fat-free lutein preparation inhibited ovulation in the laying hen, and Dunham and Riddle[5] have reported that crystalline progesterone tends to inhibit ovulation of the second of the 2 follicles constituting the usual clutch sequence of ring doves and pigeons. Previous findings in birds thus appear to be in agreement with the generally accepted conclusion that the administration of progesterone inhibits ovulation in mammals.[6,7]

Differential follicular reactivity in the hen. In the steadily laying hen each of a series of follicles is ovulated at intervals of 25 to 28 hours over a number of successive days. Ovulation of the follicle initiating a cycle occurs generally between 4:00 and 7:00 o'clock (E.W.T.) of the morning following a day of missed ovulation. This first follicle of the cycle, or clutch sequence, is more sensitive to progesterone and other ovulation-inducing substances administered under stated conditions than are subsequent follicles of the sequence.[8] We shall therefore make the following distinctions:

C_1 follicle, the first or primary follicle of a clutch sequence, normally ovulated 14 or more hours following lay of last egg of the preceding cycle, or at least 38 hours following a prior ovulation.

C_s follicle, any follicle except the C_1 follicle of a clutch sequence, typically ovulated within an hour following lay of the preceding egg, or from 25 to 28 hours following a preceding ovulation. It is not necessary, at this time, to distinguish between constituent C_s follicles.

Materials and Methods. Experimental and control material consisted of laying hens of several standard breeds, including Rhode Island and New Hampshire Reds, White Leghorns, Columbians and some crossbreds. Most of the hens were in first to third year of production. No tests were limited to hens of a single breed or age. All birds were maintained in individual cages in laying batteries, were subjected to a constant 14-hour light day and had access, *ad libitum,* to a standard laying mash. Before any bird was injected for test, the hour of lay was recorded from 8:00 a.m. through 4:00 p.m. over at least the number of days required to establish the characteristics of the individual hen's laying cycle.

All tests were based on a single injection of progesterone for effect on the single normally maturing follicle next due to ovulate at a known time. Hour of normally expected ovulation of C_1 follicles was determined by autopsy of some 75 uninjected control hens during the course of reported tests. Estimation of time of next expected normal ovulation of C_s follicles was based on a previously described procedure,[9] but control hens were autopsied regularly in confirmation of the relations involved.

Diagnosis of forced ovulation of C_1 follicles was made in most hens by palpation for presence or absence of egg *in utero,* identification of such eggs preceding by at least 6 hours the similar identification of eggs dependent on ovulation at the normal hour. Other hens were sacrificed for estimation of minimal prematurity of forced ovulation.

Practically all hens injected for effect on C_s follicles were autopsied for identification of forced ovulation in accord with tested criteria.[9] The few exceptions consisted of hens which laid prematurely the egg in the oviduct at the time of injection, and which carried *in utero,* at or before the hour of normally expected ovulation, the egg consequent upon forced ovulation.

Crystalline progesterone was administered in all but a very few tests in which a corpus luteum preparation† was injected subcutaneously with good result. Progesterone was dissolved in propylene glycol for intravenous injection (wing veins), and usually an equal volume of distilled water was added to the solution prior to injection of low dosages. Not more than 1.0 ml of propylene glycol per kilo body weight was injected into any bird, a quantity shown by control tests to have no

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[5] Dunham, H. H., and Riddle, O., *Physiol. Zool.,* 1942, **15**, 383.

[6] Makepeace, A. W., Weinstein, G. L., and Friedman, M. H., *Am. J. Physiol.,* 1937, **119**, 512.

[7] Hartman, C. G., *Sex and Internal Secretions,* 1939, Baltimore.

[8] Fraps, R. M., and Dury, A., *Anat. Rec.,* 1942, **84** (No. 4), 3.

[9] Fraps, R. M., Olsen, M. W., and Neher, B. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 308.

† "Lipo-Lutin," Parke, Davis and Co.

TABLE I.

Effect of Progesterone, 4:00 p.m. Injections, in Forcing Ovulation of Ovarian Follicles of the Hen at Least 6 Hours Prematurely.

Injection		Hens with C_1 follicles			Hens with C_s follicles		
			Ovulating			Ovulating	
Route	Level, mg	Injected, No.	No.	%	Injected, No.	No.	%
Intravenous	.01	—	—	—	10	0	0
”	.02	9	1	11.1	20	0	0
”	.05	13	4	30.8	18	0	0
”	.20	27	19	70.4	11	0	0
”	.50	9	8	88.9	6	0	0
”	1.00	10	9	90.0	7	0	0
”	1.5-5.0	—	—	—	8	0	0
”	10.0	17	10	58.8	7	1	14.3
”	15.0-40.0	—	—	—	16	1	6.3
Subcutaneous	.05	20	0	0	—	—	—
”	.125	21	4	19.0	9	0	0
”	.25	29	20	69.0	8	0	0
”	.50	25	19	76.0	15	11	73.3
”	1.0	19	18	94.7	14	10	71.4
”	2.0	11	10	90.9	7	5	71.4
”	3.0	—	—	—	13	10	76.9
”	5.0	7	7	100.0	11	8	72.7
”	10.0	10	9	90.0	10	5	50.0

effect on ovulation in the hen. Progesterone was dissolved in sesame or in corn oil for subcutaneous injection (dorsal neck region).

Results and Discussion. The main body of data, based on injection of 417 hens, is summarized in Table I. The C_1 follicle is shown to be highly reactive to progesterone administered either intravenously or subcutaneously, while the reaction of the C_s follicle differs to an extreme degree with route of injection. It is notable that a fairly constant percentage, 71 to 77, of C_s follicles ovulated prematurely following subcutaneous injection of progesterone at all levels between 0.5 and 5.0 mg.

The striking contrast in reaction of the C_s follicle with route of progesterone administration suggests that the hormone must act on the follicle over a sufficiently long time as well as at a minimally effective level in order to force ovulation prematurely, conditions satisfied by subcutaneous but not by intravenous injection. This interpretation of action on C_s follicles implies a relatively rapid elimination of progesterone from the blood stream, and supposes further that the more sensitive C_1 follicle reacts to progesterone within a relatively limited time.

Direct comparison of effects on C_1 and C_s follicles is not permissible in Table I, since time from injection to expected normal ovulation of C_1 follicles is approximately 3 hours less than is the same interval for C_s follicles. Progesterone was therefore injected at 11:00 a.m. for effect on C_1 follicles, thus substantially increasing the interval from injection to normal ovulation of the C_1 follicle over that for C_s follicles following the 4:00 p.m. injections of Table I. Intravenously injected, progesterone at the 0.20 mg level forced ovulation in 53% of 17 treated hens. Progesterone subcutaneously injected at the 0.25 mg level effected premature ovulation in 54% of 24 treated hens. These yields are sufficiently high, at the submaximal doses injected, to indicate that differences appearing in Table I with respect to C_1 and C_s follicles can be attributed only in small part to differences in intervals from injection to expected normal ovulation.

It is of interest to note that the time from administration of progesterone (intravenously, 0.2 to 0.6 mg per hen or subcutaneously, 0.5 to 1.5 mg per hen) to subsequently forced ovulation of C_1 follicles is about the same, 7 to 9 hours, required for effect on C_s follicles by anterior pituitary and equine chorionic gonadotrophins.[10] Under comparable conditions the interval from progesterone injection to forced ovulation of C_8 follicles falls between 8 and 11 hours. The details of these and other tests now in progress will be reported elsewhere.

Summary. The administration of crystalline progesterone to the common domestic hen is highly effective, under stated conditions, in causing premature ovulation of normally developing ovarian follicles. Results differ with route of injection, time from injection to expected normal ovulation, injection level, and place of the follicle in the clutch sequence. First follicles of clutch sequences are ovulable at least 6 hours prematurely in 90 to 95% of hens receiving 0.5 and 1.0 mg progesterone intravenously, or 1.0 to 10.0 mg subcutaneously. Follicles other than the first of clutch sequences are ovulable by at least 7 hours prematurity in 70 to 75% of subcutaneously injected hens at dosage levels of 0.5 to 5.0 mg progesterone. Under identical conditions the intravenous administration of progesterone is relatively ineffective. Observed intervals from injection to ovulation varied from 7 to 11 hours.

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[10] Fraps, R. M., Riley, G. M., and Olsen, M. W., Proc. Soc. Exp. Biol. and Med., 1942, **50**, 313.

14152

Effects of Adrenal and Thyroid Hormones on Water Exchange in Hypophysectomized Rats.[*,†]

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After hypophysectomy in the rat there is a transient high diabetes insipidus (d. i.) which subsides fairly rapidly and is followed by a mild chronic diuresis. If only the neurohypophysis is removed or inactivated a permanent d. i. results.[1,2,3]

It has been found that under certain circumstances anterior pituitary extract could reestablish a latent d. i. in hypophysectomized rats,[1,4‡] as it does in other species, whereas neither pituitary preparations containing primarily adrenotropic hormone, nor whole adrenal cortical extract, would do so.[4] In view of reports[5,6] that desoxycorticosterone acetate (DCA) can reinduce a polyuria in hypophysectomized rats, further observations on adrenal cortical materials are reported here,

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[*] This work was aided by a grant from the Committee on Research in Endocrinology, National Research Council.

[†] We are indebted to Dr. Erwin Schwenk, Schering Corporation, for the desoxycorticosterone acetate (Cortate) used here.

We wish to acknowledge the assistance of Mr. Bennett H. Young during part of this work.

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[2] Fisher, C., Ingram, W. R., and Ranson, S. W., *Diabetes Insipidus and the Neuro-hormonal Control of Water Balance,* Edwards Bros., Ann Arbor, 1938.

[3] Gersh, I., *Res. Publ. Assn. Nerv. Ment. Dis.*, 1939, **18**, 436.

[4] Schweizer, M., Gaunt, R., Zinken, N., and Nelson, W. O., *Am. J. Physiol.*, 1941, **132**, 141.

[‡] It has been more difficult to maintain d. i. with anterior pituitary preparations in hypophysectomized rats than in other species. We have found that different lots of crude beef anterior pituitary extract, apparently prepared in a similar manner, and equally effective as stimulants of growth and appetite, do not all have the marked diuretic effect previously reported in hypophysectomized rats.[4] This is perhaps due to variation in the amounts of antidiuretic substances contained in the extracts, although quantitative studies have not been made.

[5] Selye, H., and Bassett, L., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 272.

[6] Corey, E. L., and Britton, S. W., *Am. J. Physiol.*, 1941, **133**, 511.

together with studies on the effects of thyroid substances.

Methods. Routine technics were the same as in earlier experiments.[4] Figures quoted here are all from rats at least 4 weeks after hypophysectomy. Complete pre- and post-operative figures are available but essentially duplicate earlier findings.[4] Food intake figures are not detailed since the only change brought about by the various treatments was a 27% increase with thyroid-feeding. Oxygen-consumption was measured at weekly intervals by the method of Tainter and Rytand.[7] Urine specific gravity was determined generally twice weekly with a falling drop apparatus (Fisher) on samples specially collected to prevent contamination.

Desoxycorticosterone acetate. This material, given in 1 mg daily doses, elevated the water exchange but the responsiveness of animals varied widely. The effect was sometimes not noticeable until after one week (*cf.* [4]) and reached its maximum after 2 weeks of treatment. These results are in general agreement with those of others who obtained a more rapid and extensive response by using larger doses. The diuresis occurs in the absence of any significant change in the other factors measured, viz., food intake, body weight, specific gravity of the urine, and oxygen-consumption. These and other results are summarized in Table I.

Whole Extract of the Adrenal Cortex. The material given was a concentrate prepared in sesame oil at Princeton University. Quantitative studies of a similar preparation have been reported in other connections.[8] It was given in 0.5 cc daily doses (equivalent to 125 g fresh tissue), and treatment was continued for 3 weeks, in contrast to the earlier 7-day observations with aqueous cortical extract.[4] Again, however, no effects were obtained on water exchange or on the other variables measured here.

Thyroid-feeding. When thyroid powder was fed in doses regulated to restore the consumption of oxygen to normal (approximately 75 mg per day) there was a small but consistent rise in water intake, without an accompanying rise in urine volume. In half of the cases the urine output was if anything reduced. The absence of the expected diuresis was associated with an increase in the specific gravity of the urine. Food intake was increased. The weight losses characteristic of untreated hypophysectomized rats were exaggerated by the thyroid-treatment. Toxic symptoms were noted and several animals, not included in the tables, died during the course of the treatment.

When 0.02 mg of thyroxin was injected daily the results were similar to those obtained above, but are not included in the tables.

Since these results were possibly complicated by the toxicity of the treatments,[9] a dose of thyroxin was sought which would give some stimulation to oxygen-consumption but which would not cause serious weight losses or increase mortality. Thyroxin in 0.005 mg daily doses was found to meet these requirements. In such non-toxic doses, however, it had no effect on water exchange.

Thyroid-feeding and DCA Combined. When thyroid powder in doses which restored oxygen-consumption to normal was fed together with daily injections of 1 mg of DCA, no toxic action of the former was apparent. A water intake greater than that seen with either treatment alone occurred while the urine volume increased some but not proportionately to the water intake. The relative absence of diuresis again was associated with an elevation of the specific gravity of the urine, and under these circumstances indicates that this effect of thyroid-feeding was not due solely to the toxicity of the treatment.

Discussion. In hypophysectomized dogs several investigators have found a diuretic action of thyroid treatment,[9,10] but not in the rat.[11] Thyroxin will, however, augment the already high d. i. which follows hypothalamic lesions in the rat,[12] as in other species. Our results would imply that in the rat the

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[7] Tainter, M. L., and Rytand, D. A., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 361.

[8] Eversole, W. J., Gaunt, R., and Kendall, E. C., *Am. J. Physiol.*, 1942, **135**, 378.

[9] Keller, A. D., *Endocrinology*, 1942, **30**, 408.

[10] Bruhn, J. M., *Am. J. Physiol.*, 1942, **135**, 572.

[11] White, H. L., *Am. J. Physiol.*, 1937, **119**, 5.

[12] Clark, G., *Quart. Bull. Northwestern Univ. Med. School*, 1940, **14**, 96.

TABLE I.
Water Exchange, Oxygen Consumption, and Urine Specific Gravity in Hypophysectomized Rats Receiving Various Treatments.

Treatment	Water intake (cc)						Urine volume (cc)						O_2 consumed (cc/cm^2/hr)		
	Before treatment	During treatment			After treatment		Before treatment	During treatment			After treatment		Before treatment	During 2nd and 3rd wks	After treatment
	7 days	1st wk	2nd wk	3rd wk	1st wk	2nd wk	7 days	1st wk	2nd wk	3rd wk	1st wk	2nd wk			
DCA	37*	52	73	72	47	33	32	43	63	68	42	27			
	16	20	20		18	12	9	10	13		12	9			
	15	18	24		15	13	11	16	24		12	12			
	17	19	23		23	16	14	16	21		20	15			
	21	20	27		24	22	15	17	24		20	20			
	23	29	36	34	31	21	17	26	31	32	24	21	.50	.45	.35
	15	15	18	19	13	12	13	13	15	15	11	10	.47	.48	.52
	20	29	37	30	22	19	13	23	25	19	16	14	.39	.54	.45
	18	27	30	33	23	19	13	19	23	25	19	14	.48	.55	.54
Avg	18.	22.	27.		21.	17.	13.	18.	22.		17.	14.	.46	.51	.47
Specific gravity of urine (avg)							1.018	1.020	1.016	1.015	1.014	1.016			
Adrenal	24	20	20	22	19	19	16	15	15	15	15	16	.50	.58	
Cortical	16	13	14	12	14	14	12	12	9	7	8	11	.46	.50	
Extract	14	13	12	11	12	16	12	10	8	8	8	9	.47	.54	
	16	21	21	19	20	23	11	14	14	14	14	17	.46	.42	
Avg	18.	17.	16.	16.	16.	18.	13.	13.	12.	11.	11.	13.	.47	.51	
Specific gravity of urine (avg)							1.020	—	1.018	1.019	1.020	1.021			
Thyroid-fed	16	15	22	24	25	18	10	8	10	10	10	12		.97	
	14	15	16	17	17	10	11	9	8	8	4	7		.82	
	11	12	15	17	17	10	7	6	6	6	7	6		.99	
	19	15	16	14	14	12	15	11	6	6	6	9		.80	
	14	16	16	18	24	19	10	9	7	6	12	12		1.07	
	17	23	22	23	26	23	11	14	9	10	15	15		1.08	
	18	29	26	29	32	24	15	19	14	15	20	17		1.28	
Avg	16.	18.	19.	20.	22.	12.	11.	11.	9.	9.	11.	11.		1.00	
Specific gravity of urine (avg)							1.020	1.034	1.031	1.037	1.031	1.022			
DCA and	17	28	40	47			13	16	19	26			.54	1.18	
Thyroid-fed	22	20	25	38	38	24	14	16	14	23	21	15	.50	1.07	.60
	18	26	33	37	20	14	17	17	20	21	15	10	.47	1.04	.48
	17	21	35	38	22	18	14	17	22	23	12	12	.50	1.10	.47
	20	28	45	54	47	36	15	19	27	34	32	21	.55	.96	.68
	19	26	31	37	18	17	15	16	20	25	12	13	.49	1.04	
	22	35	42	51	30	24	18	31	36	43	23	19	.39	.91	
Avg	20.	26.	35.	43.	29.	22.	16.	19.	23.	28.	19.	15.	.49	1.04	.56
Specific gravity of urine (avg)							1.018	1.029	1.031	1.030	1.025	1.021			
Thyroxin-	23	22	21				15	13	15					.78	
injected	21	24	21				14	15	15					1.00	
(small	28	30	27				17	17	16					.72	
dose)	19	20	24				9	9	10					—	
	24	29	23				11	12	12					.97	
	33	31	27				22	16	16					.89	
	19	26	24				11	14	13					.71	
Avg	24.	26.	24.				14.	14.	14.					.84	

Average Figures on Normal, Intact Animals.
Daily water intake, 21 cc; Specific gravity of urine, 1.06.
Daily urine volume, 8 cc; Oxygen consumption cc/cm^2/hr, 1.00 ± 0.024 (St. Er.).

* This case is not included in the average because it is obviously atypical.

diuretic action of thyroid hormone is dependent upon the presence of a functional anterior pituitary.

Silvette and Britton[13] developed the hypothesis that the diuretic action of the anterior pituitary was elaborated through the adrenal cortex, and Corey and Britton demonstrated the diuretic action of DCA in short (12-hour) tests in hypophysectomized rats.[6] In experiments of longer duration Selye and Bassett[5] restored a high d. i. in hypophysectomized rats with DCA. Both groups of investigators use large doses. Ragan *et al.*[14] showed at about the same time that a d. i.-like syndrome could be induced in intact dogs with prolonged treatment with DCA—a study supplemented by Mulinos *et al.*[15]

The 1 mg doses of DCA used here were selected to see if amounts that might be considered physiological would also restore d. i. in hypophysectomized rats. The selection of a "physiological" dose was admittedly arbitrary. The 1 mg dose used was about 20 times the minimal life-maintaining dose for adrenalectomized rats in our colony. Its effects in increasing water exchange were definite but mild, and it did not produce anything like a maximal d. i. No indication of an action of this type was seen, however, when animals were treated with a whole extract of the adrenal cortex in large dosage, suggesting another difference[16] in the biological effects of the various cortical steroids.

Since the amounts of DCA needed to produce a real d.i. after hypophysectomy are large, and as the amount of this substance in cortical extracts or tissue is small,[17] the possibility cannot be ignored that its action in this respect is a pharmacological overdosage phenomenon. In any case it hardly seems likely that the adrenal cortex is the sole route through which the anterior pituitary effects its diuretic action.

Desoxycorticosterone, or related adrenal steroids, may constitute one of a complex of influences under anterior pituitary control which act together to maintain d. i. when the posterior pituitary is absent. The thyroid is probably involved in this complex, as are the pituitary factors which maintain a normal growth, activity, and appetite[18,1] and hence normal salt intake,[19] nitrogen excretion,[20] etc.

Summary. The effects of adrenal cortical and thyroid substances were studied on the water exchange and related phenomena in hypophysectomized rats during periods after the initial high postoperative d. i. had subsided. 1. Desoxycorticosterone acetate in 1 mg daily doses elevated water exchange, but not to levels characteristic of d. i., whereas whole extract of the adrenal cortex was without effect. 2. Thyroid-feeding in doses that restored oxygen consumption at least to normal elevated the water intake slightly but not the urine output. There was an associated rise in urine specific gravity. The diuretic effects of thyroid hormone under other circumstances in the rat must depend upon the presence of an anterior pituitary. 3. Non-toxic doses of thyroxin, although affecting oxygen consumption, had no effect on water exchange. 4. When desoxycorticosterone and the larger doses of thyroid were given in combination, the toxicity of the latter was overcome. Water intake was doubled, but urine volume did not increase proportionately due to the opposing action of the thyroid substance.

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14153

Development of Resistance to Penicillin by Pneumococci.

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The primary object of this study was to determine whether pneumococci develop resistance to penicillin when exposed to this anti-bacterial agent in the infected animal. As corollaries of the finding that such resistance may develop, observations were also made on (a) the capacities of different strains of pneumococcus to acquire resistance, (b) the retention of resistance when contact between organism and penicillin was discontinued, (c) the change in *in vitro* sensitivity accompanying the development of resistance *in vivo*, and (d) the response of *penicillin-resistant* pneumococci to sulfonamides. The results of these studies are presented here.

Experimental. A. *Development of resistance to penicillin in vivo.* The parent strains of pneumococcus used in this experiment were the type I McGovern and the type III CHA strains, used previously in many of our sulfonamide studies. These pneumococci were exposed to penicillin by serial passage through 7 groups of mice treated with this drug. Briefly, the procedure was as follows. In the initial experiments, 4 groups of 10 white mice (males weighing 16 to 18 g) were infected intraperitoneally, each mouse receiving 10^{-6} cc of a 12- to 14-hour blood broth culture of the desired parent strain. One group of 10 animals served as untreated controls. The animals in the remaining 3 groups were treated with 500, 1000 and 2000 Oxford unit doses of penicillin, these amounts being administered by stomach tube 2, 8 and 14 hours after infection and every 8 hours thereafter for 5 additional days.* Cultures of heart blood were prepared from 2 to 6 treated mice dying either during therapy or shortly thereafter. These cultures were incubated at 37.5°C for 12 to 14 hours and then pooled. Five-tenths cc of this pooled culture was injected intraperitoneally into an untreated mouse; a 12- to 14-hour blood broth culture prepared from the heart blood of this animal was used to infect a second lot of 40 mice, which received the same treatments as the first. This procedure was repeated for 6 consecutive passages. At the end of these transfers the effectiveness of penicillin therapy against infections with the parent strains was redetermined in order to make certain that the activity of our preparation had not gradually deteriorated during the passage period.

The results of the experiment with type III pneumococcus, CHA, (Table I) show that this strain developed considerable resistance to penicillin. Thus when mice infected with the parent organisms were treated with 1000-unit doses of penicillin, 60% recovered and those that died lived an average of 95 hours (Experiment A). In contrast to this, similar treatment afforded little if any protection to mice infected with organisms obtained from the sixth serial passage (Experiment G); none of these animals recovered and their average survival time, 32 hours, was only 4 hours longer than that of untreated controls. Even 2000-unit doses, which afforded complete protection against infections with the parent strain, gave little protection against the passaged strain (*cf.* Experiments A and G).

The type I McGovern strain also developed resistance to penicillin (Table II). Eighty per cent of the mice infected with the parent strain survived when treated with 1000-unit doses of penicillin, and the average survival time of the animals that died was 127 hours (Experiment A). In contrast to this, only 10% of the mice infected with the organisms from the sixth serial passage survived when treated similarly (Experiment G); in this case, the average survival time of the animals that died

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* The above doses represented 2.5, 5 and 10 mg of the penicillin preparation supplied us through the courtesy of the Lilly Research Laboratories, Indianapolis, Indiana. These amounts were administered in solution in 0.2 cc of 1% sodium bicarbonate.

TABLE I.

Development of Resistance to Penicillin by Type III Pneumococcus, Strain CHA: 10 Mice Infected in Each Group.

	Infecting Organisms			No. of deaths Days after infection						Avg hrs survival of mice that died	30-day survivors	
Exp.	Source	No. in infecting dose	Units P‡ per dose*	1	2	3	4	5	6-10		No.	%
A	Parent strain†	400	500	2	3	2	2	0	0	50	1	10
			1000	0	0	1	1	1	1	95	6	60
			2000	0	0	0	0	0	0	—	10	100
			0	2	8	0	0	0	0	28	0	0
B	2 mice of Exp. A treated with 500 units of P; 2 mice with 1000 units	640	500	1	8	1	0	0	0	35	0	0
			1000	0	4	3	0	0	1	67	2	20
			2000	0	0	0	0	0	1	143	9	90
			0	0	10	0	0	0	0	30	0	0
C	5 mice of Exp. B treated with 1000 units of P	280	500	1	9	0	0	0	0	31	0	0
			1000	0	2	3	1	0	0	57	4	40
			2000	0	0	1	0	0	0	69	9	90
			0	1	9	0	0	0	0	28	0	0
D	5 mice of Exp. C treated with 1000 units of P; 1 mouse with 2000 units	490	1000	0	6	4	0	0	0	45	0	0
			2000	0	1	2	3	1	1	82	2	20
			0	0	10	0	0	0	0	33	0	0
E	4 mice of Exp. D treated with 1000 units of P; 2 mice with 2000 units	970	1000	1	9	0	0	0	0	33	0	0
			2000	0	1	2	2	0	0	65	5	50
			0	5	5	0	0	0	0	29	0	0
F	4 mice of Exp. E treated with 2000 units of P	270	1000	2	7	1	0	0	0	34	0	0
			2000	0	3	4	1	1	0	58	1	10
			0	5	5	0	0	0	0	28	0	0
G	6 mice of Exp. F treated with 2000 units of P	1070	1000	0	10	0	0	0	0	32	0	0
			2000	0	5	2	1	0	0	50	2	20
			0	2	8	0	0	0	0	28	0	0
Control	Parent strain	600	500	0	5	1	1	1	0	54	2	20
			1000	0	0	3	1	0	0	88	6	60
			2000	0	0	0	0	0	0	—	10	100
			0	6	4	0	0	0	0	26	0	0

* Doses of penicillin administered 2, 8 and 14 hours after infection and at 8-hour intervals thereafter for 5 additional days.

† This parent strain was of maximum virulence and invasiveness, having been passed through normal mice either daily or on alternate days for more than 4 years.

‡ P = penicillin.

was only 51 hours. Although this comparison shows that strain McGovern acquired considerable resistance to penicillin, this organism did not develop this property as rapidly as did strain CHA (compare Experiments D in Tables I and II), nor did it develop as great a degree of resistance as did the CHA strain (compare Experiments G in Tables I and II).

B. *Retention of Resistance to Penicillin.* Cultures of the resistant CHA strain of pneumococcus, prepared from 4 mice of Experiment G (Table I), were pooled and passed daily through normal mice, the organisms isolated from the 30th serial passage being tested for their response to penicillin. In this test, groups of 10 mice were infected as described above. One group received 1000-unit doses of penicillin as indicated previously; a second group received 2000-unit doses, and a third group served as untreated controls. All 10 control mice died, the average survival time being 27 hours. All 10 mice treated with 1000-unit doses of penicillin died, their average survival time being 40 hours. Two of 10 mice receiv-

TABLE II.

Development of Resistance to Penicillin by Type I Pneumococcus, Strain McGovern: 10 Mice Infected in Each Group.

	Infecting Organisms			No. of deaths Days after infection						Avg hrs survival of mice that died	30-day survivors	
Exp.	Source	No. in infecting dose	UnitsP‡ per dose*	1	2	3	4	5	6-10		No.	%
A	Parent strain†	460	500	0	6	3	0	0	1	62	0	0
			1000	0	0	0	0	0	2	127	8	80
			2000	0	0	0	0	0	1	157	9	90
			0	1	9	0	0	0	0	35	0	0
B	5 mice of Exp. A treated with 500 units of P	970	500	0	3	2	2	0	1	70	2	20
			1000	0	1	0	0	0	3	129	6	60
			2000	0	0	0	0	0	0	—	10	100
			0	3	7	0	0	0	0	31	0	0
C	5 mice of Exp. B treated with 500 units of P	29	500	0	6	2	0	0	1	57	1	10
			1000	0	0	1	4	0	1	100	4	40
			2000	0	0	0	0	0	0	—	10	100
			0	1	9	0	0	0	0	35	0	0
D	4 mice of Exp. C treated with 1000 units of P	620	500	6	0	2	0	0	1	55	1	10
			1000	0	0	2	2	4	2	106	0	0
			2000	0	0	0	0	0	0	—	10	100
			0	0	9	1	0	0	0	38	0	0
E	5 mice of Exp. D treated with 1000 units of P	510	1000	1	6	1	1	0	0	43	1	10
			2000	0	0	0	0	0	0	—	10	100
			0	3	7	0	0	0	0	25	0	0
F	5 mice of Exp. E treated with 1000 units of P	890	1000	0	1	5	0	2	0	74	2	20
			2000	0	0	0	0	0	2	161	8	80
			0	1	9	0	0	0	0	32	0	0
G	2 mice of Exp. F treated with 2000 units of P	980	1000	0	5	3	1	0	0	51	1	10
			2000	0	2	0	0	1	2	105	5	50
			0	1	9	0	0	0	0	32	0	0
Control	Parent strain	1630	500	0	2	1	2	1	1	80	3	30
			1000	0	0	1	0	0	1	106	8	80
			0	2	8	0	0	0	0	31	0	0

* Doses of penicillin administered 2, 8 and 14 hours after infection and at 8-hour intervals thereafter for 5 additional days.

† This parent strain was of maximum virulence and invasiveness, having been passed through normal mice either daily or on alternate days for more than 4 years.

‡P = penicillin.

ing 2000-unit doses recovered; those that died survived 59 hours on the average. From a comparison of these results with those of Experiment G (Table I), it is clear that the resistance to penicillin developed by strain CHA was not significantly impaired by as many as 30 consecutive passages through normal mice. Whether a larger number of passages would alter this characteristic has not been determined.

C. *Comparison of the* in vitro *sensitivity of the parent and resistant strains to penicillin.* Quantities of 9 cc of basal medium, containing 2% defibrinated rabbit blood and various concentrations of penicillin,† were inoculated with 1 cc quantities of 10^{-5} dilutions of 12-hour blood broth cultures of the respective parent

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† The basal medium was a beef heart infusion broth (pH 7.8) containing 2% neopeptone and 0.5% sodium chloride. Various amounts of penicillin, dissolved in 5% $NaHCO_3$ (and sterilized by filtration through sintered glass), and 2% defibrinated rabbit blood were added just before use. The amounts of penicillin added were such that the final concentrations (after addition of inoculum) were 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 Oxford units per cc of medium.

TABLE III.
Comparison of Growth of Parent and Resistant Strains of Pneumococcus in Media Containing Penicillin.

Strain	Inoculum: No. of pneumococci per cc culture	Hrs incubated	Growth in medium containing penicillin Units per cc					
			0	0.2	0.4	0.8	1.6	3.2
III CHA Parent	730	12	+	+	+	—	—	—
		24	+	+	+	—	—	—
		48	+	+	+	—	—	—
III CHA Resistant	530	12	+	+	+	+	?	—
		24	+	+	+	+	+	—
		48	+	+	+	+	+	—
I McGovern Parent	750	12	+	+	+	—	—	—
		24	+	+	+	—	—	—
		48	+	+	+	—	—	—
I McGovern Resistant	830	12	+	+	+	+	—	—
		24	+	+	+	+	+	—
		48	+	+	+	+	+	—

and resistant organisms.‡ The resulting cultures, incubated at 37.5°, were examined visually for growth at 12, 24 and 48 hours. The observations of growth, recorded in Table III, show that the strains of pneumococcus that had developed resistance to penicillin *in vivo* were significantly less sensitive to the *in vitro* action of this anti-bacterial agent than were the parent strains. The highest concentration of penicillin permitting visible growth of either parent strain was 0.4 unit per cc of medium. Comparable growth of either resistant strain occurred even when the penicillin concentration was 1.6 units per cc.

D. *Response of* penicillin-resistant *pneumococci to sulfapyridine.* A group of 25 mice was infected as described above with the *penicillin-resistant* McGovern organisms isolated from the mice of Experiment G (Table II). Ten of these animals served as untreated controls; the remaining mice received 10 mg doses of sulfapyridine, suspended in acacia and administered by stomach tube 2, 8 and 14 hours after infection and every 8 hours thereafter for 5 additional days. A second group of 25 mice, infected with the parent McGovern organisms, was treated in an identical manner.

The results of this experiment showed that infections with penicillin-resistant pneumococci responded to sulfapyridine at least as well as infections with the parent strain. All 15 mice infected with the resistant strain recovered under sulfapyridine therapy, as compared with recovery of 10 of the 15 mice infected with the parent strain. All control animals of both groups were dead within 36 hours after infection.

Discussion. It is now well established that pneumococci and other microörganisms may acquire resistance to the sulfonamides upon prolonged exposure to these drugs. This reaction occurs in the treated patient[1–4] as well as in experimental animals and culture media. In treated patients, the reaction does not commonly occur during ordinary short-term usage of the sulfonamides.[4] It does occur frequently, however, during prolonged administration of these drugs,[4] and definitely militates against successful therapy of diseases which require long-term therapy, such as pneumococcal endocarditis, unresolved pneumonia and, in some instances, meningitis. Obviously, in such conditions it would be highly desirable to have available a therapeutic agent to which organisms did not develop resistance as readily as to the sulfonamides.

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‡ The resistant organisms used in this test were the strains isolated from mice of Experiments G (Tables I and II).

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From the results of the present study it is apparent that pneumococci may develop resistance to penicillin upon continued exposure to this anti-biotic agent in the treated animal. McKee and Rake[5] observed a slight increase in the resistance of pneumococci to penicillin following serial passage in culture media containing this drug. Rammelkamp and Maxon[6] and Abraham and co-workers[7] also found that staphylococci acquired resistance to penicillin *in vitro*. Superficially, at least, these observations indicate that prolonged penicillin therapy suffers from the same defect as extended sulfonamide treatment. This suggestion is borne out by the apparent clinical failure of penicillin therapy[8] in subacute bacterial endocarditis caused by *Streptococcus viridans,* a disease invariably requiring long-term treatment.

The present work has also shown that development of penicillin resistance does not alter the sulfonamide sensitivity of pneumococci. Powell and Jamieson[9] and McKee and Rake[5] have previously shown that the converse is also true; namely, that development of sulfonamide resistance does not alter sensitivity of pneumococci to penicillin. These observations are of theoretical interest in that they indicate that the fundamental actions of these two agents are distinctly different. Practically, they offer the hope that alternate short-term treatment with these substances might succeed in curing those infections which because of development of resistant organisms have hitherto resisted treatment with either agent alone.

Finally, it has been shown that the two strains of pneumococcus used in this study differed significantly in the rates at which they acquired resistance to penicillin, the CHA strain becoming resistant more rapidly than the McGovern strain. It is noteworthy that these strains developed resistance to sulfapyridine at different rates,[10,11] the CHA strain also acquiring resistance to this drug more rapidly than the McGovern strain. A study of the composition of the parent strains showed that the organisms composing the CHA strain were more variable and unstable in respect to sulfonamide sensitivity than were the organisms of the McGovern strain.[12] The present observations suggest that the same variability and instability characterize the reactions of the CHA strain to penicillin.

Summary. The present study has shown the following: (1) Two strains of pneumococcus developed resistance to penicillin as a result of serial passage through mice treated with this drug. (2) The rate at which resistance developed and the degree of resistance acquired varied significantly with the different strains. (3) The resistance of the one strain tested was not impaired by 30 serial passages through normal mice, indicating that, once established, resistance to penicillin is retained for a considerable period. (4) The development of resistance to penicillin *in vivo* was accompanied by an increase in resistance to this drug *in vitro*. (5) The response of the pneumococci to sulfonamides was not altered by the development of resistance to penicillin.

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14154

Dicumarol (3,3′-Methylene-Bis-(4-Hydroxy-Coumarin)) in Rats with Impaired Liver or Kidney Function.

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Extensive research has been carried on in recent years concerning the use of the anticoagulating agent, Dicumarol, as a substitute for Heparin.[1,2] Of the numerous problems to be investigated before such a substance can be used with safety in clinical medicine, not least in importance is the effect of its use in pathological cases. It is the purpose of this paper to report the effects of administering Dicumarol to rats with impaired liver or kidney function.

While our work was in progress, Bollman and Preston[3] reported that dogs in which liver damage had been induced by the administration of carbon tetrachloride, or in which kidney impairment was produced by subcutaneous injections of uranium acetate showed a markedly increased sensitivity to Dicumarol.

In this paper we report similar experiments, done on rats instead of dogs, since there is a difference between dogs and rats in susceptibility to Dicumarol.[4] Furthermore, our experiments were done over relatively long periods of time on a large number of animals in an attempt to show quantitative differences in response to Dicumarol at different stages of impairment of these organs.

Procedure. In all cases albino rats weighing 125-175 g were kept on a special diet recommended by Overman, Field, Baumann and Link[5] because of the more consistent results obtained in their observations with Dicumarol over those of ordinary stock diets. Blood samples were collected by cardiac puncture, and the plasma diluted with saline to a 12.5% concentration. A modification[6] of the Quick method was used in making the prothrombin determinations. The dosage of Dicumarol was 2.5 mg given by stomach tube in a suspension of .5% gum acacia. In nearly all cases blood collections were made 24 hours after the administration of Dicumarol, this time interval being used because in the rat it has been demonstrated to be the point of maximal effect.[4]

Liver damage was produced by subcutaneous injection of .2 cc/kg or .5 cc/kg carbon tetrachloride. These injections were made daily in a large number of rats. A part of these animals were used as controls and their blood prothrombin time determined in intervals of one or more days. The other part was fed 2.5 mg Dicumarol once after having been treated for several days with carbon tetrachloride. Prothrombin time was determined 24 hours later.

Fig. 1 shows the result in the form of a graph. Every point on the curve represents the average prothrombin time of groups of 6 to 15 animals except the last 2 determinations at 14 days, with .5 cc/kg carbon tetrachloride, where only 2 and 5 animals were still alive. The graph clearly demonstrates that the injections of carbon tetrachloride alone produce only a mild, if any, elevation of the prothrombin time, even after administration is continued for as long as 14 days.

The observations of previous investigators[7,8]

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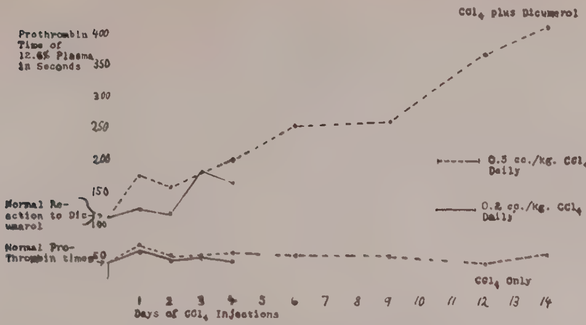

FIG. 1.
Effect of One Dose of 2.5 mg Dicumarol in Rats after Repeated Subcutaneous Injections of CCl_4.

as well as our own histological examinations prove that only a few injections of this dose were adequate to produce definite liver damage. Our findings, as represented in the graph, confirm those of others[9,10] in that the injured liver alone, unless such injury is very extensive, does not appreciably change the prothrombin time. The upper curves of the graph demonstrate how animals treated with carbon tetrachloride react to Dicumarol with a much more intensive depression of prothrombin level than with animals without liver damage. This increased sensitivity occurs earlier with higher doses of carbon tetrachloride and becomes more pronounced with the increasing number of carbon tetrachloride injections.

One notices on all 4 curves that the prothrombin time on the second day is slightly below the value on the first day. One may speculate whether this is due to a transitory stimulation of prothrombin formation.

It is also interesting that carbon tetrachloride-treated rats show an increased response to Dicumarol at a time when their prothrombin level is normal or only slightly elevated.

In another series of experiments, we tested the effect of kidney impairment upon the action of Dicumarol. Unilateral or bilateral nephrectomy was performed on rats. In the control group we found that the nephrectomy caused no change from normal in prothrombin time over a period of 4 days, at the end of which the rats died. Unilateral nephrectomy failed to change the reaction of such animals to Dicumarol. The bilaterally nephrectomized group which received Dicumarol immediately after the operation showed the same degree of prothrombin time elevation within 24 hours as normal animals do. However, 48 hours after the administration of Dicumarol, instead of going down as usual, the prothrombin time had increased from an average of 120 seconds to 229 seconds. In 72 hours it had reached 270 seconds. (See Table I.) These results would indicate that complete loss of kidney function greatly increases the effect of Dicumarol.

It might be argued that the accumulation of urea could affect the Dicumarol action on prothrombin time. In a limited number of experiments we found that 1.5 g of urea administered simultaneously with Dicumarol had no greater influence on prothrombin time than Dicumarol alone would have.

Since the exact mode of action and the fate of Dicumarol in the body is not entirely known, no complete explanation of the above-described findings can be given. However, it appears that liver damage interferes with the reserve power of the liver to produce needed prothrombin. As long as that damage is mild, the prothrombin level can obviously be sustained at about normal values. But the additional depressing effect of Dicumarol results in a marked lowering of the blood prothrombin content.

The results in nephrectomized animals would indicate that the kidney apparently is

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TABLE I.
Effects of Double Nephrectomy on Prothrombin Time in the Rat Before and After Administration of Dicumarol by Stomach Tube.
Prothrombin Time in Seconds.

Without Dicumarol				After Dicumarol (2.5 mg)				
No. of animals	Days after nephrectomy	Range, sec.	Avg, sec.	No. of animals	Days after nephrectomy	Time after Dicumarol (hrs)	Range, sec.	Avg, sec.
8	2	33-43	38	4	1	24	85-175	117
3	4	39-43	41	6	1	24	62-225	120
			—	10	1	24	60-188	130
		Avg	40	6	3	24	82-145	107
				7	1	24	100-154	128
							Avg	120
				7	3	48	98-367	197
				7	2	48	150-348	234
				10	2	48	103-540	256
							Avg	229
				6	3	72	107-465	270
							Avg	270

not predominantly concerned with the metabolic effect of Dicumarol, since the depression of prothrombin level after 24 hours is the same in normal and nephrectomized animals. However, it must be assumed that Dicumarol or an active split product leaves the body by way of the kidney. This would explain the continued and increased depression of the prothrombin level in nephrectomized animals beyond the time in which a return to normal occurs in intact rats.

Summary. Experiments in rats are described in which the effect of gradually increasing liver damage upon the action of Dicumarol was studied. Injections of carbon tetrachloride which do not significantly influence the prothrombin level greatly sensitize rats to Dicumarol. Bilateral nephrectomy results in a prolonged and increased effect of this drug. These findings indicate the necessity of care in the administration of this drug to patients having even a mild degree of liver damage or suffering from impairment of the kidney function.

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Effect of Shock on the Small Blood Vessels of the Ear of the Rabbit.

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Peripheral vasoconstriction has been thought to be one of the cardinal manifestations of developing shock if not the initiating factor, whether shock be produced by hemorrhage[1] or by trauma.[2,3] It has been demonstrated[4] that stimulation of the sympathetic nervous system produces a train of effects strikingly similar to those associated with traumatic shock. Also it has been found[1] that during the production of shock by repeated hemorrhage the peripheral blood flow is more greatly reduced in the intact animal than in the sympathectomized animal. It has been our object in this study to observe directly the differences in the responses of the peripheral blood vessels when the nerve supply is intact and when it is interrupted. Since the transparent chamber inserted into the ear of the rabbit has been shown recently by us[5] and previously by others,[6,7] to be suitable for physiologic studies, we have employed this method.

Method. For this study we used healthy young albino rabbits weighing between 2 and 4 kg. The chambers were fully vascularized in each instance. Observations were made on 7 animals. In 4 of them the innervation of the ear was intact; in the remaining 3 the ear had been denervated as described previously.[5] Shock was produced by intestinal manipulation with the animal under the influence of pentobarbital sodium as has been done in dogs.[8] Observations of the vessels were made with a compound microscope, a magnification of ×200 being used. Photographs of the vessels were taken at half hour intervals coinciding with the periods of intestinal manipulation.

Results. The average length of time required for these animals to go into shock was about 3 hours. Death usually ensued within about an hour after the onset of definite manifestations of shock or after the systolic blood pressure had declined to about 60 mm of mercury.

In the innervated ear the earliest change noted in the behavior of the peripheral vessels was a spasmodic constriction of the arterioles. As this continued, the period of relaxation of the arterioles subsequent to constriction became less and less. As the period of onset of shock was approached there were phases during which the blood flow in the chamber was completely shut off. As this train of events proceeded, the quantity of blood flowing in the vessels became less. Within a few minutes prior to death the arteriolar walls became relaxed and blood flow ceased.

In the denervated ear the blood vessels behaved in exactly the same fashion with one exception. At no time during the experiments did we observe maximal constriction with complete cessation of the blood flow in the denervated ear. In this group of animals there was rather a gradual progressive constriction of the arterioles until the final relaxation just prior to death.

In both groups of animals the functional capillaries diminished in number in proportion to the degree of constriction of the arterioles. At no time was there evidence of capillary dilatation. A striking feature of the peripheral

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[*] Fellow in Medicine.

[1] Freeman, N. E., Shaffer, S. A., Schecter, A. E., and Holling, H. E., *J. Clin. Invest.*, 1938, **17**, 359.

[2] Blalock, Alfred, and Cressman, R. D., *Surg., Gynec. and Obst.*, 1939, **68**, 278.

[3] Tomb, J. W., *South African M. J.*, 1941, **15**, 109.

[4] Cressman, R. D., and Benz, E. W., *Arch. Surg.*, 1939, **39**, 720.

[5] Levinson, J. P., and Essex, H. E., *Am. J. Physiol.*, in press.

[6] Abell, R. G., and Page, I. H., *J. Exp. Med.*, 1942, **75**, 305.

[7] Sanders, A. G., Ebert, R. H., and Florey, H. W., *Quart. J. Exp. Physiol.*, 1940, **30**, 281.

[8] Pender, J. W., and Essex, H. E., *Anesthesiology*, in press.

circulation was the diminution of the amount of blood in the venules as the experiment progressed.

Comment. From these experiments it has been shown that constriction of the arterioles in the ear of the rabbit during the production of shock by trauma can occur in the denervated as well as in the innervated ear. The question immediately arises whether there is a release of some vasoconstrictor substance during the production of shock. By administration of doses of ergotoxine sufficient to block the vasoconstrictor action of epinephrine it has been shown[9] that there was not a reduction of blood volume such as occurred when epinephrine was given alone.[10] It has also been shown[11] that an animal whose sympathetic nervous system has been paralyzed by ergotamine tartrate closely resembles a sympathectomized animal. Since the blood vessels of one group of our animals did not possess a sympathetic nerve supply it is logical to assume that the vasoconstriction observed was owing to the liberation into the blood stream of some vasoconstrictor substance. Whether this was epinephrine or some other substance is not known. The kidney, adrenals and pancreas have recently[12] been eliminated as factors in this peripheral constriction. The possibility that the vasoconstriction was the result or accommodation of the vascular bed to a decreased blood volume was considered. This was thought an inadequate explanation, since the vasoconstriction occurred early and much before a great change of blood volume was considered probable.

An increased number of impulses from the nerve of a traumatized limb has been demonstrated by Slome and O'Shaugnessy,[13] who expressed the belief that this barrage of nerve impulses is of fundamental importance in an understanding of the causation of surgical shock. In our experiments reflex production of peripheral vasoconstriction, we believe, has been eliminated by the complete interruption of the nerve supply to the vessels observed. Of course the possibility of a release of sympathin through nervous barrage from the site of trauma still exists as well as the remote effects of local capillary damage.[14] Others[2] have stated that it is very doubtful if nervous impulses play as important a role as has been ascribed[13] to them.

The observation of temporary cessation of flow in the innervated ear, as contrasted with the steady but progressive narrowing of the vessels in the denervated ear, is interesting. The apparently constant, although diminished, flow in the denervated ear may lend some support to the observations which have been made that the peripheral flow is maintained better in sympathectomized animals than in those that have intact nervous systems during the production of shock.[1,11]

Summary. A study has been presented of the reactions of the small blood vessels of the ear of the rabbit during the production of shock by intestinal manipulation. This study was made on four animals whose ears were innervated and on three whose ears were denervated.

Conclusions. In the rabbit marked peripheral vasoconstriction occurs during the production of shock but it is not entirely dependent on an intact nerve supply. However, the nature of the peripheral vasoconstriction is somewhat altered by denervation.

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[9] Freeman, N. E., *Am. J. Physiol.*, 1933, **103**, 185.

[10] Erlanger, Joseph, and Gasser, H. S., *Am. J. Physiol.*, 1919, **49**, 345.

[11] Schlossberg, T., and MacKay Sawyer, M. E., *Am. J. Physiol.*, 1933, **104**, 195.

[12] Abell, R. G., and Page, I. H., *Am. J. M. Sc.*, 1942, **205**, 309.

[13] Slome, David, and O'Shaugnessy, Laurence, *Brit. J. Surg.*, 1938, **25**, 900.

[14] Zweifach, B. W., *Am. J. Physiol.*, 1941, **133**, 501.

14156 P

Acrolein and Shock. Possible Relationship of Lipoid Breakdown Products to Shock Associated with Burns.*

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The causative factor in shock associated with burns is still questionable. Many theories have been proposed and more than 20 substances have been listed as having a part in its production. Yet no one substance has been demonstrated in the blood or tissues of patients suffering from this condition to prove definitely that it is the cause.

Many investigators who adhere to the toxic theory assume that breakdown products of *proteins* are responsible for the peripheral circulatory failure and capillary damage which are perhaps the most important factors in producing the condition of shock. This concept may be true but the possibility that similar breakdown products of body fats may play an equally important role should not be entirely disregarded.

It is well known that when fats or other glycerol esters are heated directly, a certain amount of the glycerol portion of the radical is changed to acrolein, a very toxic substance. Since fats are normally present in all tissues of the body, it appears likely that acrolein may be produced when tissue is subjected to burns, especially if the burn extends to the subcutaneous fat.

In order to test the validity of this assumption, experiments were undertaken to demonstrate first, whether acrolein itself might produce the picture of shock in animals; and second, whether the causative agent in shock produced by burns might be identified as, or linked with, acrolein or its breakdown products. This paper is a preliminary report of the first part of these studies.

Laboratory Procedure. Employing 10 dogs (wt. 4-8 kg), 2 cats (wt. 3.5-3.7 kg), and one rabbit (wt. 2.7 kg), the following procedure was used: acrolein[†] diluted in normal salt solution to a concentration of 1:10 was injected in one or another manner; subcutaneously, intraperitoneally or intravenously in single or divided dosage. Total dosage of acrolein ranged from 0.1-0.5 ml (One animal received 2.33 ml in divided doses). In addition 3 control animals (dogs) were run with ether anesthesia alone, 2 of them for comparable periods of 4 to 5 hours. The third was maintained under anesthesia for 18 hours.

Careful kymographic records of blood pressure levels by means of a cannula in the carotid artery, as well as pulse rate and respiration were kept. Hematocrit readings were taken at frequent intervals for evidence of fluid shift with associated hemoconcentration. The femoral vein was used for injections and for removal of blood samples. Ether anesthesia was maintained through a tracheal cannula. All animals were examined pathologically at autopsy and their tissues studied microscopically.

Results. All the reactions were acute, the animals surviving from one to 5 hours, an average of 3 hours after the initial injection of acrolein. Hematocrit readings consistently showed some degree of hemoconcentration even in the brief period of these acute experiments, with a maximum increase of 12% of cells. All animals clinically presented the classical picture of shock with hemoconcentration, a falling blood pressure and terminal pulmonary edema. Pulse and respiration rates were less regularly altered. Pathologic examination both grossly and microscopically showed confirmatory evidence of shock, with intense pulmonary edema, and visceral con-

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* Acknowledgment is hereby expressed to the members of the Departments of Pathology, Pharmacology, Physiology, Chemistry, Bacteriology and Immunology for their interest, encouragement and cooperation.

[†] The acrolein used was obtained from the Eastman Kodak Company.

gestion even to the point of capillary hemorrhage, especially in the mucosa of the stomach and duodenum. The spleen less regularly presented the classical picture of constriction and pallor, due we believe to the acute nature of the experiments. One animal injected subcutaneously (0.37 ml) showed almost complete hemolysis. The 3 control animals with ether anesthesia alone showed an average hemoconcentration of less than 5%. Pathologically, their tissues were normal.

Summary and Conclusions. Acrolein injected into experimental animals by any of several routes produces a condition identical in its clinical and pathological manifestations to that generally accepted as "shock." By using the intravenous route and relatively large doses in certain animals, it is believed that this picture is the direct result of capillary damage by acrolein or lipoid breakdown products, rather than the result of the liberation of "H" substance or other tissue breakdown protein substances. Studies concerning the development of antibodies to acrolein in the serum of burned animals and individuals are in progress.

14157

Action of Bacterial Toxins on Tumors. IV. Distribution of Tumor-Hemorrhage Agents Among Bacterial Species.*

S. H. HUTNER AND PAUL A. ZAHL.

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In a survey[1] ranging over 28 genera and nearly 100 strains of bacteria it was found that bacterial substances capable of inducing hemorrhage in implanted tumors were restricted to gram-negative species. A close correlation between the occurrence of hemorrhage-producing agents and the toxic somatic O antigens of gram-negative bacteria was adduced. Further investigations demonstrated a close parallelism between the lethal and the tumor-hemorrhage properties of purified bacterial products[2] and a parallel inhibition of both of these actions by sulfonamide drugs.[3]

Since the O antigens of gram-negative bacteria (and the chemically similar Vi antigens) have been identified in several recent studies with the essential immunizing constituents of antibacterial vaccines, it was considered of interest to explore further, in both pathogenic and non-pathogenic bacteria, the distribution of toxins inducing the type of vascular damage which we believe to be characteristic of the O and Vi antigens. The present study is an extension of the original survey, and includes the testing for tumor-hemorrhage activity of an additional 30 gram-negative and 7 gram-positive species.

Methods. The hemorrhage activity of concentrated bacterial cultures was tested on white male Rockland mice bearing 7-day-old implants of Mouse Sarcoma 180. In preparing mice for assay, a 2-3 mm cube of tumor tissue was implanted subcutaneously near the axilla and allowed to grow for 7 days, by which time it had become well vascularized and had developed into a spherical mass about 1 cm in diameter. The material to be assayed was administered intraperitoneally. The bacteria were killed by addition of a small quantity of toluene-chloroform mixture. This treatment is unlikely to inactivate the O antigen.[4] Where necessary the volume was reduced by pervap-

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* We wish to express our gratitude to Prof. C. B. Van Niel, who supplied most of the cultures used in this survey.

[1] Zahl, Paul A., Hutner, S. H., Spitz, S., Sugiura, K., and Cooper, F. S., *Am. J. Hyg.*, 1942, **36**, 224.

[2] Zahl, Paul A., and Hutner, S. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 116.

[3] Zahl, Paul A., and Hutner, S. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 285.

[4] Ungar, J., Jenner, R. M., and Hunwicke, R. F., *J. Path. and Bact.*, 1942, **54**, 331.

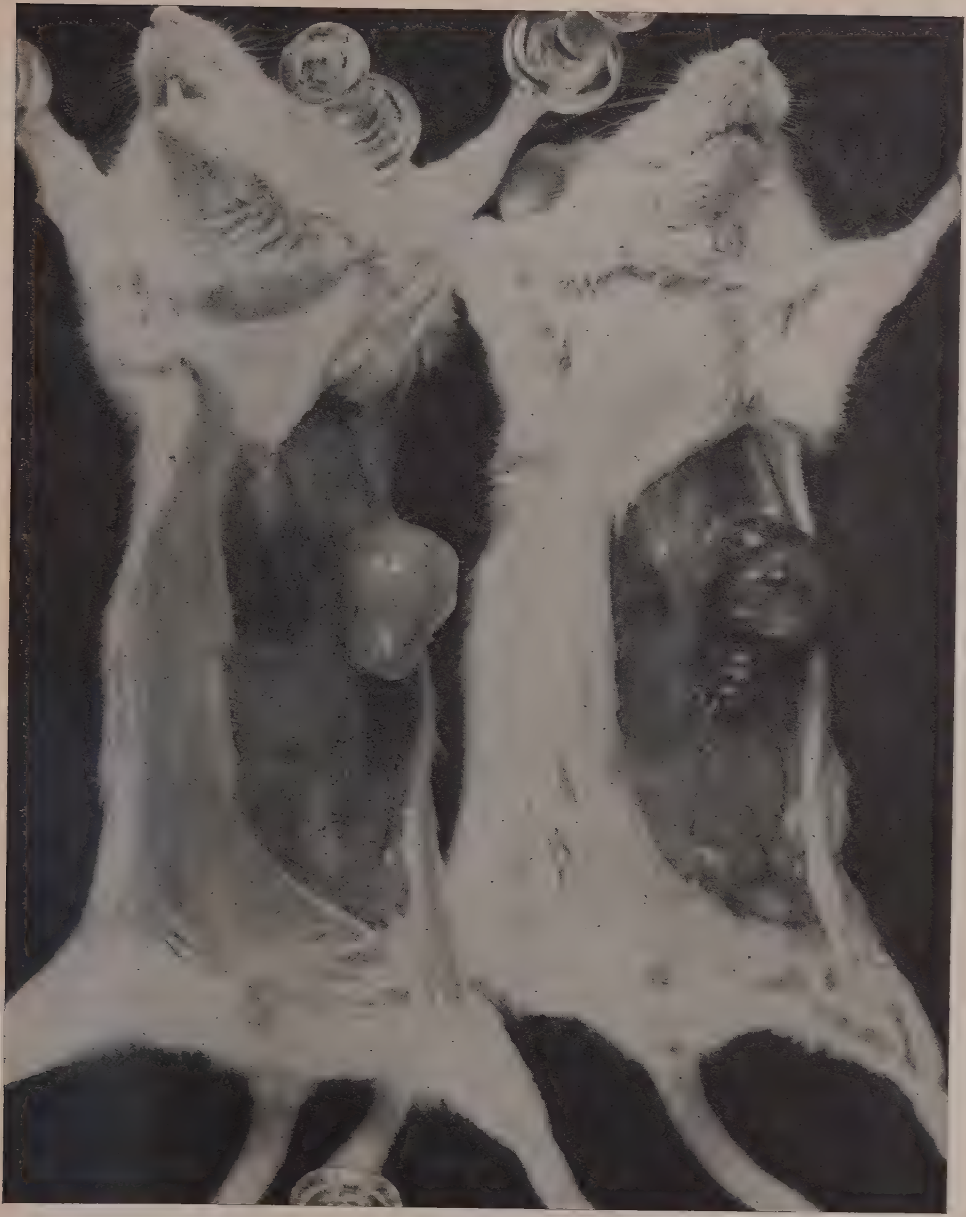

FIG. 1.

Comparison of Normal and Hemorrhagic Tumors.

Left: Mouse with normal seven-day-old Sarcoma 180.

Right: Mouse with hemorrhagic seven-day-old Sarcoma 180. Hemorrhage was induced by the intraperitoneal injection of dead *Salmonella typhosa* suspension about six hours previous to dissection.

oration through cellulose tubing.[†] An effort was made to inject doses approaching the lethal limit while keeping the volume injected to less than 0.5 ml. Hemorrhage in tumors reaches its maximum within 8 hours after injection of the toxin. The animals at this stage were sacrificed, and the tumors dissected. The degree of hemorrhage was graded as slight (*), considerable (**), and marked (***). Normal tumor tissue is pink; a hemorrhagic tumor is deep red to deep purple (Fig. 1).

Table I lists the bacterial species thus tested. For comparative purposes, the bacterial genera tested in the original survey are listed below.[‡]

Discussion. If these toxins, which are generally associated with pathogenicity, are also to be found in non-pathogenic species, then it becomes increasingly probable that the hemorrhage-inducing substance is characteristic of a larger class of organisms, namely, the gram-negative bacteria. Therefore, in the present extension of the initial survey, special attention was paid to a number of non-pathogens and in particular to photosynthetic bacteria and to the *Pseudomonas* complex of species considered by Stanier and Van Niel[5] to represent a relatively unspecialized group.

An examination of Table I will disclose an interesting example of the toxicity of a presumably unspecialized and non-pathogenic bacterium, *Rhodospirillum rubrum.* This photosynthetic gram-negative purple bacterium gave marked tumor-hemorrhage.[§] This, coupled with the widespread occurrence of tumor-hemorrhage agents in the *Pseudomonas* group, may be regarded as a strong indication that tumor-hemorrhage toxins are conservative evolutionary characters not necessarily associated with a pathogenic tendency; still, the possession of these toxins suggests that with the addition of invasiveness a harmless gram-negative bacterium may be converted into a pathogen.

It seems reasonable to suppose that tumor-hemorrhage toxins, whether found in pathogens or non-pathogens, are similar; and presumably identical with those ordinarily observed during infections by gram-negative bacteria.[‖]

Decision as to whether the hemorrhage factor is invariably bound up in a complex O antigen on the *Shigella-Salmonella* model would require separate investigation of each species.

Since it has been shown repeatedly that rough strains lack the O antigen, it is obvious that unless attention is paid to the possibility of smooth-rough transformation, misleading results are likely to be obtained with stock cultures of unstable species. This may explain why *Salmonella typhosa* strains tested in our initial survey did not induce hemorrhage, whereas the virulent Panama strain of *S. typhosa* tested in the present study was highly hemorrhagic. Similarly, *Pasteurella cuniculicida* elicited hemorrhage, whereas *P. boviseptica* and *P. cuniculicida* had been previously reported inactive.[6] Inasmuch as a comparative study of tumor-hemorrhage as a function of the presence of O antigens may supply an

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[†] Purchased from the Sylvania Industrial Corporation, New York City.

[‡] Gram-Negative	Gram-Positive
Aerobacter (hem.)	*Bacillus* (no hem.)
Brucella (hem.)	*Actinomyces* (no hem.)
Chromobacterium (hem.)	*Clostridium* (no hem.)
Escherichia (hem.)	*Corynebacterium* (no hem.)
Hemophilus (hem.)	*Gaffkya* (no hem.)
Klebsiella (hem.)	*Mycobacterium* (no hem.)
Neisseria (hem.)	*Pneumococcus* (no hem.)
Pasteurella (no hem.)	*Sarcina* (no hem.)
Proteus (hem.)	*Staphylococcus* (no hem.)
Pseudomonas (hem.)	*Streptococcus* (no hem.)
Salmonella (hem.)	
Serratia (hem.)	
Shigella (hem.)	
Vibrio (uncertain)	

[5] Stanier, R. Y., and Van Niel, C. B., *J. Bact.,* 1941, **42**, 437.

[§] According to Stanier and Van Niel[5] all photosynthetic bacteria are gram-negative. We have found this true of the photosynthetic purple bacteria *Streptococcus varians* and *Rhodobacillus palustris,* neither of which however induced tumor-hemorrhage. It should be noted that *Streptococcus varians* appears to be a rod rather than a streptococcus in the usual morphological sense, and that *Rhodobacillus palustris* is not a spore-former.

[‖] The simultaneous presence of exotoxins, as in *Shigella dysenteriæ,* some strains of *E. coli,* etc., must of course be taken into consideration.

[6] Gardner, R. E., Bailey, G. H., and Hyde, R. R., *Am. J. Hyg.,* 1939, **29** (Sec. B), 1.

TABLE I.

Species	Source of Cultures	Effect of bacterial suspensions on tumors Numerator = degree of hemorrhage Denominator = No. of animals tested		
Gram-Negative:				
Aerobacter aerogenes	Van Niel	***/3	**/1	
” *cloacæ*	” ”	***/5		
Acetobacter sp.	” ” strain 2.1	***/2	**/1	*/2
Alcaligenes (bacterium) radiobacter	” ”	***/7	**/2	
Brucella abortus	H. L. Gilman, N. Y. State Veterinary Coll. Phenolized vaccine	***/5	**/5	
Hydrogenomonas sp.	Van Niel	***/3	**/1	
Neisseria catarrhalis	ATCC 7900	***/2	**/3	
Pasteurella cuniculicida	” 7277	***/3	**/2	
Phæobacterium sp.	Van Niel	***/6	**/2	0/1
Phæomonas varians	” ”	*/1	0/7	
Protoaminobacter alboflavus b	” ”	***/4	**/2	
Pseudomonas acidovorans	” ”	***/6	**/1	*/1
Ps. caryocyaneus	” ”	***/4		
Ps. droebachense (marine agar digester)	” ”	***/3	**/1	
Ps. indoloxidans	” ”	***/3	**/2	0/1
Ps. iridescens (marine agar digester)	” ”	***/2	**/2	
Ps. ovalis	” ”	***/5		
Ps. punctata	” ”	***/5		
Ps. savastanoi	” ”	***/3	**/2	
Ps. stutzeri	” ”	***/3	**/1	*/1
Ps. sp. (marine nodule-former)	” ”	***/3	**/1	*/1
Ps. (Hydrogenomonas) sp. (not identical with *Hydrogenomonas* above)	” ”	***/2	**/2	
Rhizobium meliloti	ATCC 4401	0/5		
Rhodobacillus palustris (photosynthetic)	Van Niel	0/11		
Rhodospirillum rubrum (photosynthetic)	” ”	***/7	**/6	*/1
Salmonella typhosa (Panama strain)	G. F. Luippold, U. S. Army Med. School	***/5	**/2	
Spirillum sp. (marine)	Van Niel	***/2	**/2	*/1
Streptococcus varians (photosynthetic)	” ”	0/8		
Vibrio beijerincki (marine agar digester)	” ”	***/2	**/2	
Vibrio tyrogenus	ATCC 7085	***/3	**/2	
Gram-Positive:				
Aerobacillus polymyxa	Van Niel	0/7		
Erysipelothrix muriseptica	ATCC 4162	0/6		
” *rhusiopathiæ*	” 805	0/5		
Listeria monocytogenes (1)	” 984	***/3	**/1	*/1
” ” (2)	” 4428	***/1	**/1	0/1
” ” (ovine) (3)	P. Olafson, N. Y. State Veterinary Coll.	0/5		
Micrococcus cyaneus	Van Niel	0/7		
Rhodococcus agilis	” ”	0/7		
Sporosarcina ureæ	” ”	0/6		
Non-Bacterial:				
Cytophaga krzemieniewskæ	” ”	0/5		

ATCC = American Type Culture Collection.
*** = marked hemorrhage.
** = considerable hemorrhage.
* = slight hemorrhage.
0 = no hemorrhage.

independent test of the postulated relation between the O antigens and the tumor-hemorrhage agents, we propose to examine this point in detail later.

We are unable at the present time to account for the absence of a demonstrable toxin in *Rhizobium*, *Phæomonas*, *Streptococcus varians* and *Rhodobacillus palustris*.

The two isolates of *Listeria* are the only gram-positive organisms observed to yield hemorrhage-toxins. The taxonomic position of this species is uncertain, yet *Erysipelothrix*, to which *Listeria* may be closely related,[7,8] was inactive. The anomalous systematic position of *Listeria* has been recognized by Stanier and Van Niel.[5]

The lack of hemorrhage with *Cytophaga krzemieniewskæ* is not conclusive as only small amounts of cell body were available. A study of more material and other members of the *Myxobacteriales* is needed to determine whether the gram-negativity of this group is indicative of a relationship to gram-negative bacteria, if tumor-hemorrhage constitutes a criterion.

Conclusions. An extension was made of an earlier survey of bacterial substances inducing hemorrhage in implanted mouse tumors. It was found that with a few exceptions gram-negative forms induced tumor-hemorrhage, while this property, with one exception, was lacking in the gram-positives. An interesting example was the induction of hemorrhage by the photosynthetic gram-negative purple bacterium *Rhodospirillum rubrum*. It is concluded that toxins inducing tumor-hemorrhage, and probably bound up in the complex O antigens, are characteristic of gram-negative bacteria.

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[7] Barber, Mary, *J. Path. and Bact.*, 1939, **48**, 11.

[8] Hutner, S. H., *J. Bact.*, 1942, **43**, 629.

14158

Lung Tumors Following Intraperitoneal Injection of 1:2:5:6-Dibenzanthracene into Young Mice of Three Strains.

CLARA J. LYNCH.

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Data have already been published[1] demonstrating inherited differences in degree of susceptibility to spontaneous and to tar-induced lung tumors in two inbred strains of mice when the criteria for susceptibility included not only the percentage of tumor-bearing animals per group but also the number of tumor nodules per individual. The same criteria revealed constitutional differences in susceptibility among 3 strains of mice observed after intraperitoneal injections of 1:2:5:6-dibenzanthracene into very young animals.[2] The present paper reports further data obtained after intraperitoneal injections, combined with the original observations made following this technic.

The three strains used, the Swiss, our branch of the Bagg albinos, and No. 1194,* differ in susceptibility to spontaneous neoplasms. The Swiss have the highest lung tumor incidence. In a sample of 448 mice (Table I), about one-half, or 46.1%, had tumor. This figure is based upon mice of all ages. However, tumor incidence increases with age, and, in groups of mice of this strain that have reached a year and a half or more, 80% have tumor. Tumors occur early in this strain, a growth having been observed in a mouse only 3 months of age. One of the characteristics of lung tumor is that the nodules may be multiple. In the Swiss strain, of those animals that have tumor, almost half have multiple nodules, the largest number of nodules observed in a single

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[1] Lynch, C. J., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 186.

[2] Lynch, C. J., *Cancer Research*, 1941, **1**, 740.

* Source of material: Swiss-8, (8117, 8123); Bagg-L (1916, 1917); Strain No. 1194, (421, 453).

TABLE I.
Spontaneous Lung Tumor in 3 Strains of Mice.

Strain	No. of mice all ages	Lung tumor %	Earliest occurrence of tumor	Largest No. nodules per mouse
Swiss	448	46.1	3 mos.	13
Bagg	1382	26.3	5 mos.	9
1194	2660	4.6	11 mos.	7

individual being 13. The Bagg strain is less susceptible. Among 1382 animals of all ages, about a quarter, or 26.3%, had tumor. The growths do not appear quite so early—the youngest mouse to be found with tumor was 5 months old, and the largest number of nodules in any mouse was 9. The least susceptible of the 3 strains is No. 1194. Of 2660 mice of all ages, only 4.6% had lung tumors. Very few tumors are found in mice less than a year old. In this sample, only one mouse had tumor as early as 11 months. Even in old mice the incidence is low; it is only 17% in mice between 2½ and 3 years of age. Of the few animals with tumor, the large majority have a single nodule, and 7 nodules is the largest number found in one individual. In general, in the strains with the highest tumor incidence, tumors appear earlier and the number of nodules per individual is higher.

To induce lung tumors, a single intraperitoneal injection of 0.5 minim of a 0.4% solution of 1:2:5:6-dibenzanthracene in olive oil was given to mice 2 to 4 days old. The variation in age was due to an attempt to have the animals approximately the same size. This dose of 0.125 mg of dibenzanthracene is a large one for such small specimens and is probably the most effective for tumor induction. Counts were made of the nodules seen on the surface of the lung with a dissecting microscope.

The results of the injection are shown in Fig. 1. It is apparent that the method used is effective in inducing large numbers of tumor nodules. Mice from each of the strains, dying within a few days of the injection were not autopsied and are not recorded on the chart. A few representatives of each strain died before the 60th day and were autopsied, but none had tumor. Between the 65th and 75th day after injection, 12 mice of the Swiss strain died or were killed. All were found to have tumor, the number of nodules per mouse ranging from 24 to 86. This is a much higher number than ever observed to arise spontaneously in these strains. No individual of the Swiss strain, coming to autopsy after this time was tumor-free. A number of mice belonging to the 3 strains died during the next 3 months and between the 180th and 210th day all remaining animals either died or were killed. In the Swiss strain the number of nodules per mouse had increased enormously, some animals having more than 150 tumor foci. In the upper ranges it is difficult to make accurate counts because the nodules coalesce, and the figures given here represent minimal values. Of 31 mice examined during the last 35 days of the experiment, only one individual had fewer than 84 foci, and the majority had many more. Of the Bagg strain, not as many animals were available for injection with the hydrocarbon. Autopsies of the few mice that died between the 110th and 160th day showed that all had lung nodules but not as many nodules per individual as found in the Swiss during the same period. Of the 12 Bagg mice that died or were killed during the last 35 days, all had tumor, and all but one had between 27 and 61 foci per mouse. Strain 1194 presented a very different picture. Of the 29 mice autopsied between the 175th and 210th day, only half (15) had tumor and no mouse during the entire experiment had more than 5 nodules.

It is evident from inspection of the last 35 days of the chart that in spite of a slight amount of overlapping, the mice of these 3 inbred strains fall into 3 distinct groups. The greatest spread is seen in the Swiss, less in the Bagg, and least in strain 1194. The first 2 strains mentioned both attained an incidence of 100% tumor mice and if the percentage of mice affected were the only criterion of susceptibility these 2 stocks would appear to be alike, but when susceptibility is judged by the num-

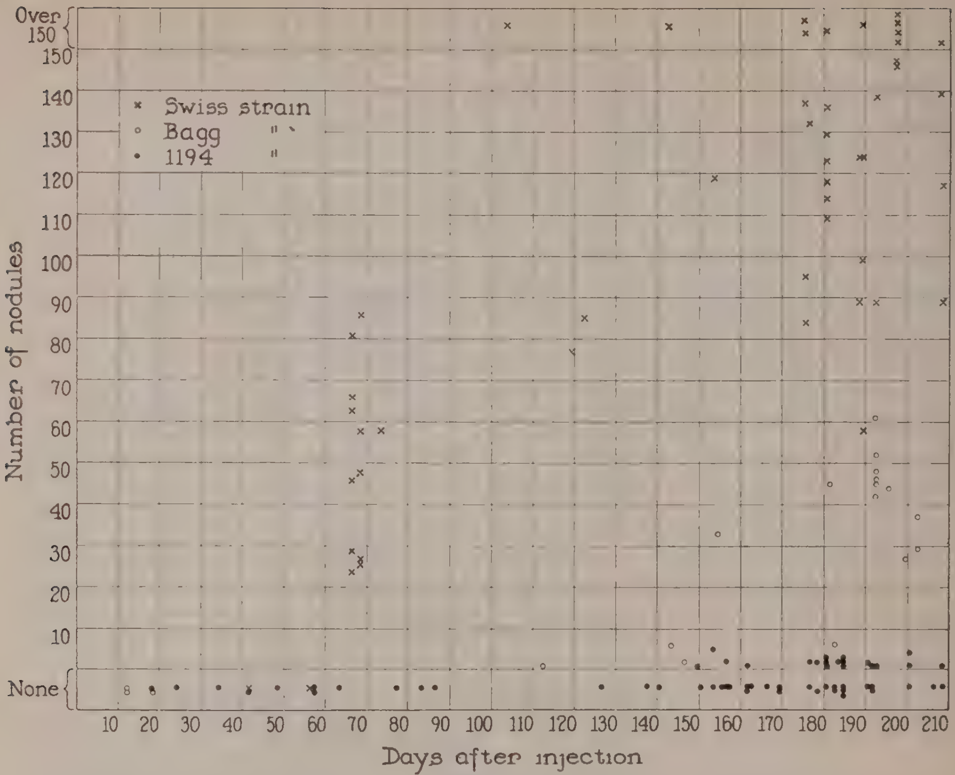

FIG. 1.

Showing the number of lung nodules per individual in mice of 3 strains injected intraperitoneally when 2-4 days old with 0.5 minim of a 0.4% solution of 1:2:5:6-dibenzanthracene in olive oil.

ber of nodules per individual strain differences are still apparent. The third strain, 1194, attained an incidence of 51.7%. Thus it is seen that the induced tumors appeared at a much earlier age than do the spontaneous growths, and they appeared in a much higher percentage at 6 to 7 months than ever observed in this strain at any age under ordinary laboratory conditions. However, the range of nodules per individual was no greater, in fact not quite as great, as it is in the spontaneous disease.

Since the differences noted are presented by three inbred strains, it is clear from the experimental results that differences in degree of susceptibility are inherited and that a single pair of genes could not account for the observed facts. At least 3 genes must be involved, and a larger number is not excluded. That a number of genes are concerned in the inheritance of susceptibility to induced lung tumors has been found by Heston also. In the genetic analysis of the results of crossing certain of his strains of mice, it was shown that at least 4 and probably more pairs were indicated,[3] and 2 susceptibility factors were located on 2 different chromosomes.[4] Andervont's studies[5] also have indicated more than one pair. The genetic relationships of our strains are being investigated further.

Summary. A single intraperitoneal injection of 0.5 minim of a 0.4% solution of 1:2:5:6-dibenzanthracene in olive oil was given to mice 2 to 4 days old from three inbred strains. On the basis of the percentage of tumor mice that resulted, and the number of lung tumors induced in each individual, it is concluded that the degree of susceptibility to induced lung tumor is inherited, and that 3 or more genes must be involved.

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[3] Heston, W. E., *J. Nat. Cancer Inst.*, 1942, **3**, 69.

[4] Heston, W. E., *J. Nat. Cancer Inst.*, 1941, **2**, 127.

[5] Andervont, H. B., *J. Nat. Cancer Inst.*, 1940, **1**, 135.

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Erratum

Volume 51, No. 3, p. 369, Article 13979, by F. A. Beach on Progesterone, second column, last paragraph, "sexually receptive male" should read "sexually active male."

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